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Contents

Original Article

Role of Viral Protein-Protein Interactions and Possible Targets for New Therapeutics

Rehan Zafar Paracha, Fahad Perviez, Babar Aslam, Ayesha Obaid, Sidra Qureshi
Sobia Manzoor, Kaneez Fatima, Ishtiaq Qadri
Atta-ur-Rahman School of Applied Biosciences
rehanzfr@gmail.com, rehanzfr@ncvi.nust.edu.pk

Abstract

Viral infections are the cause of serious infirmities in humans and kill millions of people every year. Management of viral diseases is one of the challenges faced by the whole world which needs improvement in prevention and treatment options. Complete understanding of the consequences of viral proteins interaction network on host physiology is essential. Towards this goal, deciphering viral protein-protein interactions is one of the perspective which can help in our understanding about the basis of viral pathogenesis and the development of new antivirals. Indeed, viral infection network based on viral-viral proteins will provide an elusive and investigative framework to articulate rationalize drug discovery based on proteomics scale of viruses. In this study, proteomics a collection of viral-viral protein interactions reporting different studies of hepatitis C virus, Influenza A virus, Dengue virus and SARS Coronavirus. Our effort of protein-interactions was focused on different studies reporting interactions between viral proteins encoded by the viruses under study. The study is integrated with a broad and original literature-curated data of viral-viralprotein (197 non-redundant) interactions.

Introduction

Viruses interfere vital cellular processes, including cell growth, gene expression, differentiation and signalling pathways by perturbing the networks of cellular regulatory processes. Alongside, the capture of cellular machinery by viruses for the production of their own progenies and evasion from the host immune system is the major cause of pathogenesis. Mechanisms essential for this sabotage of cellular physiology facilitated by viral proteins can be understood only by uncovering that how viral proteins disturb normal cell signalling networks along with the significance of intra-play of viral protein-protein interactions.

Viral protein–protein interactions are necessary for the fundamentals of virion assembly and its egression from the cell. Often, these interactions cause functional perturbations which may lead to complex diseases, like cancer(Ahmed and Heslop, 2006; Hebner and Laimins, 2006).

In this context, we have reported the analysis and combination of viral protein-protein interactions. Based on an extensive scientific literature search, we provided a resource of manually curated interactions between viral proteins.

Hepatitis C Virus (HCV)

Since the discovery of this virus as the causative agent of hepatitis(previously known as Non-A, Non-B hepatitis) (Choo *et al*., 1990), significant improvement has been made in understanding of the molecular biology of virus and its replication pathways inside the cell. However, the progress has been hindered by non-efficient cell culture system of virus (Barth *et al*., 2006). HCV is a member of Flaviviridae family. It is an enveloped, positive-strand RNA virus with 9.6kb genome (Rice, 1996). Un-translated region (UTR) is present at 5' and 3' ends, which flanks single poly protein consisting of 3010 amino acids. It is further processed into 4 structural proteins i.e. core (C) , Envelope (E1, E2) and p7 along with 6 non-structural (NS) proteins comprising NS2, NS3, NS4A, NS4B, NS5A and NS5B. These proteins are post processed by host and viral proteases(Lohmann *et al*., 1996) for their cleavage as individual functional proteins. HCV genome gets translated into a large polypeptide that has to be cleaved into appropriate proteins to induce viral replication. In a study, it was observed that at the N-terminus of NS3 is a serine protease that cleaves the polypeptide at four distinct regions i.e. NS3/NS4A, NS4A/ NS4B, NS4B/ NS5A and NS5A/ NS5B. NS4A forms the integral part of NS3 and this association is strongly mediated by Isoleucine (Ile)-29 of NS4A. In addition, two residues of NS4A i.e. Ile-25 and Val-23 are involved in developing its association with NS3 as well as with NS4B and NS5A (Dimitrova *et al*., 2003).

Structural proteins C, E1and E2 serve as structural component and play their part in the assembly of virions (Bartosch *et al*., 2003; Nielsen *et al*., 2004; Yasui *et al*., 1998). P7 is a small protein and considered to be a membrane channel protein(Carrere-Kremer *et al*., 2002). Non-structural proteins consist of NS2–NS5B.Their proteolytic cleavage is mediated by two viral proteases. NS2-NS3 protease, which cleaves NS2 from the poly protein. NS3 serine protease along with its cofactor 4A mediates the cleavage of other proteins of virus. N-terminus of NS3 constitutes serine protease domain while its C-terminus consist of RNA Helicase domain. NS4A acts as a cofactor for NS3 serine protease (Tellinghuisen and Rice, 2002). NS4B function is still not clear but it is proposed that it is associated with membranes and helps in

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replication of virus. NS5A is hydrophilic membrane associated protein whose function is unknown, until recently genetic evidence has been provided that in the phase of HCV RNA replication, NS5A performs two distinct functions. A cis-acting and other one is trans-acting function. Cis-acting happens as part of the HCV replication complex, and trans-acting function may occur outside of the replication complex. It was confirmed by an inhibitor of NS5A, BMS-790052(Fridell *et al*., 2011). The NS5B protein is the RNA-dependent RNA polymerase, helping in the replication of the virus.

Several studies have shown that viral non-structural proteins are also involved in replication, virion assembly and its maturation, especially in case of Flaviviridae family (Kummerer and Rice, 2002; Liu *et al*., 2003). NS proteins interact with themselves and structural proteins during viral lifecycle. These interactions are of significant values in terms of development of specific inhibitors by analysing the residues present at the interface which are involved in interaction.

Several studies report the interactions among HCV proteins. Here provided a review of these interactions reported in literature showing the interactions among viral proteins and their significance in relation to viral replication and assembly.

Interaction between the Core protein and the helicase portion of NS3 has been confirmed recently. The protease portion of NS3 does not play any role in it. This interaction was confirmed by four different biochemical methods. Protein-protein interaction could be interrupted by two types of inhibitors including SL201 and core106 which is a truncated core protein from C-terminus. Cross-linking experiments suggested that the interaction of core-NS3h is most likely driven by C protein oligomerization. SL201 is basically involved in the production blockage of infectious virus, and does not take part in the production of subgenomic HCV replicon. Experiments have also articulated that SL201 has no role in the inhibition of virus entry. This data reveals the importance of core and viral helicase interaction in the assembly of virion (Mousseau *et al*., 2011).

HCV core protein forms a nucleocapisd that gets surrounded by a lipid bilayer. The mutational analysis of HCV core protein has been determined that indicates that hydrophilic domain (1-115) of this protein is critically involved in its multimerziation which, in turn, is involved in various functions of the core protein (Matsumoto *et al*., 1996). In order to determine the core-core protein interaction, several truncations were done. This led to the findings that a tryptophan rich sequence region ranging from 82-102 in the N-terminal region is important for homotypic interactions while C-terminus hydrophobic region shows a weak heterotypic interaction with N-terminal region of the core protein (Nolandt *et al*., 1997).

In addition to the interaction of core protein with itself and some other glycoproteins of the virus. It also displays interactions with the RNA dependant RNA polymerase i.e. NS5B, as determined by immunoflourescence and immunoprecipitation assays. These two proteins co-localizes onto the ER membrane. However, the C-terminal region of NS5B is critically involved in the complex formation between these two proteins as the truncated NS5B is vulnerable to establish a core protein-NS5B complex(Uchida *et al*., 2002).

NS5B forms a complex with NS3 region in the presence or absence of NS4A (cofactor for NS3 serine protease). It was shown that amino terminal of NS3 is responsible for the interaction with NS5B as both proteins were co-localized, proved by double staining analysis. It also revealed that NS4A does not interfere with NS5B and NS3 interaction. Co-immunoprecipitation assays proved that NS5B also forms a complex with NS4A in the absence of NS3. These results suggested that NS5B, NS3 and NS4A form a complex which might function as a part of replication machinery (Ishido *et al*., 1998).

NS5A modulates the HCV replication being a part of the replication complex. It was established by glutathione S-transferase (GST) pull-down and Co-immunoprecipitation assays that bacterial recombinant NS5A interacts with NS5B. Two regions,105–162and 277–334, were found to be important for their interactions (Shirota *et al*., 2002).

The strongest protein interactions among viral proteins have been found for NS3 with itself. Yeast two hybrid system was used to find these interactions. Minimal region of 174 amino acids at N-terminus of the helicase region was required for interaction which was validated by randomly introducing mutations and then narrowing down the functional interactions. The association of NS3 with itself was confirmed by Co-immunoprecipitation assays. Other parts of the NS3 protein subsidise to the stability of the NS3-NS3 interactions (Khu *et al*., 2001).

X-Ray crystallographic structure of NS5A has revealed the dimer formation for this protein in two crystal structures; NS5A (33-202) or NS5A (25-215). Both dimer structures support the concept of membrane-bound NS5A proteins associating through contact of domain I surfaces. Further studies are needed to elucidate the role of these dimer formations in HCV lifecycle (Love *et al*., 2009).

Folding and dimerization analysis of E1 and E2 proteins revealed that E1 folding is more efficient and faster than E2. E2 starts it's folding after getting associated with E1 protein. Co-immunoprecipitation and sedimentation rate analysis verified the association between these two proteins which exist as a non-covalent heterodimer, a functional subunit of HCV virion Envelope (Brazzoli *et al*., 2005).

The structural genes of HCV encode three proteins i.e. Core (C), two envelope proteins (E1) and (E2). Immunoprecipitation experiments revealed that Core protein interacts with the E1 protein in the presence of anti-core antibody. These proteins co-localize on the cytosolic region of endoplasmic reticulum (ER) membrane where E1 faces the core protein. In another study, core and glycoproteins of HCV (E1 and E2/NS1) was inserted into baculovirus expression system and each protein was inserted into a separate construct. The infected insect cells showed that core protein was phopshorylated and transported into nucleus. Deletion of C-terminal region containing hydrophobic residues, E2 protein was produced as soluble protein but not the secreted one. Contrary, there

Table 1. List of HCV protein-protein interactions.

S #	Viral Protein 1		Viral Protein 2	
	NCBI Reference Sequence	Protein	NCBI Reference Sequence	Protein
1.	NP_751919	core protein	NP_751919	core protein
2.	NP_751919	core protein	NP_751920	E1 protein
3.	NP_751919	core protein	NP_751927	NS5A protein
4.	NP_751919	core protein	NP_751928	NS5B RNA-dependent RNA polymerase
5.	NP_751920	E1 protein	NP_751921	E2 protein
6.	NP_751923	NS2 protein	NP_751923	NS2 protein
7.	NP_751923	NS2 protein	NP_751925	NS4A protein
8.	NP_751923	NS2 protein	NP_751926	NS4B protein
9.	NP_751923	NS2 protein	NP_751927	NS5A protein
10.	NP_751923	NS2 protein	NP_751928	NS5B RNA-dependent RNA polymerase
11.	NP_751923	NS2 protein	NP_803144	NS3 protease/helicase
12.	NP_751925	NS4A protein	NP_751925	NS4A protein
13.	NP_751925	NS4A protein	NP_751926	NS4B protein
14.	NP_751925	NS4A protein	NP_751927	NS5A protein
15.	NP_751925	NS4A protein	NP_751928	NS5B RNA-dependent RNA polymerase
16.	NP_751926	NS4B protein	NP_751926	NS4B protein
17.	NP_751926	NS4B protein	NP_751927	NS5A protein
18.	NP_751926	NS4B protein	NP_751928	NS5B RNA-dependent RNA polymerase
19.	NP_751926	NS4B protein	NP_803144	NS3 protease/helicase
20.	NP_751927	NS5A protein	NP_751927	NS5A protein
21.	NP_751927	NS5A protein	NP_751928	NS5B RNA-dependent RNA polymerase
22.	NP_751927	NS5A protein	NP_803144	NS3 protease/helicase
23.	NP_751928	NS5B RNA-dependent RNA polymerase	NP_751928	NS5B RNA-dependent RNA polymerase
24.	NP_803144	NS3 protease/helicase	NP_751925	NS4A protein
25.	NP_803144	NS3 protease/helicase	NP_751928	NS5B RNA-dependent RNA polymerase
26.	NP_803144	NS3 protease/helicase	NP_803144	NS3 protease/helicase
27.	NP_751921	E2 protein	NP_803144	NS3 protease/helicase
28.	NP_751920	E1 protein	NP_751927	NS5A protein

is no effect of deleting C-terminal region of E1 on its solubility (Lo *et al*., 1996).

HCV replicates itself onto the ER membrane in the presence of viral assembly mainly consisting of all non-structural proteins. It was demonstrated that all of the HCV NS proteins get sequestered on the cytosolic membrane of ER and produces a high concentration of viral proteins, sufficient to induce pathogenesis in vitro as well as in vivo by using Glutathione pull down assay and co-immunoprecipitation assays(Dimitrova *et al*., 2003).

Previous studies (Dimitrova *et al*., 2003; Drummer and Poumbourios, 2004; Dubuisson *et al*., 1994; Flajolet *et al*., 2000; Goh *et al*., 2001; Khu *et al*., 2001; Lanford *et al*., 1993; Lim *et al*., 2006; Lin *et al*., 1997; Lo *et al*., 1996;

Ma *et al*., 2002; Matsumoto *et al*., 1996; Molenkamp *et al*., 2003; Nolandt *et al*., 1997; Op De Beeck *et al*., 2000; Piccininni *et al*., 2002; Shirota *et al*., 2002; Uchida *et al*., 2002; Welsch *et al*., 2007; Yan *et al*., 1998) have provided valuable data on the HCV protein-protein interactions listed in Table1.

Dengue Virus

Dengue virus belongs to the family Flaviviridae that has seven non-structural proteins i.e NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Dengue virus has an RNA dependant RNA polymerase (NS5) that specifically interacts with its own Non-structural protein-3 (NS3), a helicase. These multi-domain proteins show interactions both *in vitro* and *in vivo*. However, NS3 has to compete with a cellular protein i.e. nuclear transport receptor importin–β to interact with N-terminal region of NS5 (residue 320-368). By using two hybrid system, it was observed that the virally infected cells hyperphosphorylate NS5 that preferentially interacts with nuclear localization signals like nuclear transport receptor importin β but not with NS-3. Later, the competition between NS3 and importin –β for NS5 was confirmed by using pull down assays that depicted the potential role of importin β to pull down cytoplasmic proteins to nucleus during viral infection (Johansson *et al*., 2001).

The NS3 protein of the Dengue virus has strong interactions with other NS proteins of the virus to facilitate its own helicase activity and favour viral replication. In this study, the interaction between NS3 and NS4B was identified by using two hybrid system followed by the confirmation with pull down assay. It was observed that NS-4B helps NS-3 to detach from ssRNA and improve its helicase activity *in vitro* (Umareddy *et al*., 2006).

To unravel the structural organization of flaviruses like Dengue virus and yellow fever virus, their capsid proteins were characterized. The secondary structure of their capsid proteins revealed large number of alpha helices at the C-terminus with the largest alpha helix of 20 residues. In addition, C-terminus is amphipathic while N-terminal region was not shown to be involved in structural integrity to the capsid protein (Jones *et al*., 2003).

The core region of Dengue virus type- 2 is protected with nucleocapsid region which, in turns, is surrounded with a lipid bilayer derived from the membrane (M) and an envelope protein. The mutational analysis of this membrane protein revealed that Histidine (H) at 39 position plays a pivotal role in viral replication, morphogenesis, cell entry and secretion (Pryor *et al*., 2004).

In a study, the mechanism of entry for Dengue virus (type-2) into the host cells was determined and observed that NS1 has two glycosylation sites i.e. Asn 130 and Asn 207. By inserting mutagenized cDNA into the Simian virus 40, it was observed that removal of one or both of these glycosylation sites can't abolish the dimerization and secretion of NS-1 yet secretion is greatly reduced by removing Asn 207 (Pryor and Wright, 1994).

The large genome of Dengue virus gets translated into multiple proteins that interact with each other to facilitate viral entry, replication and host specific pathogenesis. In order to find out the reason for dengue virus infection in humans but not in mosquitoes, antiNS-3 antisera was used that favoured the formation of NS-3 fragment of 50 kDa size with the N-terminal region containing 460 residues. This fragment is only formed in the dengue virus infected human but not in the mosquitoes. Further examination of this fragment revealed that there is conserved cleavage sequence within the helicase domain of NS-3, which is

Table 2. List of Dengue virus protein-protein interactions.

	Viral Protein 1			Viral Protein 2		
S#	**Virus Type**	**NCBI Reference Sequence**	**Protein**	**Virus Type**	**NCBI Reference Sequence**	**Protein**
1	Dengue virus type 1	NP_722460	envelope protein	Dengue virus type 1	NP_733807	membrane glycoprotein precursor
2	Dengue virus type 2	NP_739587	ATPase	Dengue virus type 2	NP_739590	NS5
3	Dengue virus type 2	NP_739589	ns4b protein	Dengue virus type 2	NP_739587	ATPase
4	Dengue virus type 2	NP_739591	capsid protein (C)	Dengue virus type 2	NP_739591	capsid protein (C)
5	Dengue virus type 2	NP_739582	prM (M) protein	Dengue virus type 2	NP_739583	E protein
6	Dengue virus type 2	NP_739584	NS1 protein	Dengue virus type 2	NP_739584	NS1 protein
7	Dengue virus type 2	NP_739586	ns2b protein	Dengue virus type 2	NP_739587	ATPase

cleaved by NS-2 and favours the viral replication and disease progression (Arias *et al*., 1993).

Previous studies (Arias *et al*., 1993; Brooks *et al*., 2002; Clum *et al*., 1997; Courageot *et al*., 2000; Johansson *et al*., 2001; Jones *et al*., 2003; Kuhn and Rossmann, 1995; Kuhn *et al*., 2002; Leung *et al*., 2001; Lindenbach and Rice, 1999; Ma *et al*., 2004; Niyomrattanakit *et al*., 2004; Preugschat and Strauss, 1991; Pryor *et al*., 2004; Pryor and Wright, 1994; Rey *et al*., 1995; Umareddy *et al*., 2006; Wang *et al*., 2004; Welsch *et al*., 2007) have provided valuable data on the Dengue virus protein-protein interactions listed in Table 2.

Influenza A Virus

Influenza virus A (IAV) is a member of orthomyxoviridae family. The IAV genome is 13.5kb long single stranded negative sense RNA that constitutes 11 proteins. Two surface glycoproteins; HA (haemagglutinin) and NA (neuraminidase), Non-structural proteins; non-structural 1 (NS1), non-structural 2 (NS2), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP) and three polymerase proteins; PB1(polymerase basic protein 1), PB2, and PA (polymerase acidic protein), some members also produce PB1-F2, a small protein (Ghedin *et al*., 2005). Based on the antigenic nature of surface glycoproteins HA and NA, several subtypes of IAV have been characterized. These proteins are responsible for the most antigenic variations in the virus. So far, 16 different subtypes of HA (H1-16) and 9 different subtypes of NA (N1-9) have been identified (Fouchier *et al*., 2005).

The optimum propagation of IAV in the upper respiratory tract of humans has been seen at the temperature of 33°C and this temperature is 41°C in the intestinal tract of birds. The adaptation of virus to the host is performed by the viral RNA polymerase complex comprises of PA, PB1 and PB2. The activity of IAV RNA polymerase is optimum under the temperature control of PB2.PB1 interacts with PB2 either in the presence or absence of polymeraseacidic protein. Co-expression and co-immunoprecipitation assays have proved this association by using monospecific antibodies. Two regions at the NH2 terminus of PB1 have been found, which can interact in an independent manner with PB2 to form stable complexes. C-terminal of PB1 is not involved in making any interaction with PB2. Mutational analysis further established that the interacting regions of PB1 include amino acids (aa) 48-145 and 251-321 (Biswas and Nayak, 1996).

In another study, results suggested that the 78 aa of N-terminal and residues 506-659 of PB1 were interactively involved with compartments of polymerase. Mammalian cell two-hybrid assays has also proved that N-terminal of PB1 is responsible for its interaction with the PA subunit while the PB1 C-terminal region is responsible for binding with PB2 subunit (Gonzalez *et al*., 1996). No direct interaction between PA and PB2 has been confirmed, but a three dimensional structure of a recombinant influenza virus RNP created by electron microscopy proposed that contacts among the three polymerase subunits were present (Area *et al*., 2004). The effect of PA on the nuclear accumulation of PB1 was studied using Co-expression analysis. It was suggested that PB1 must interact with PA for its efficient nuclear accumulation (Fodor and Smith, 2004).

Some other interactions reported in studies (Biswas *et al*., 1998; Biswas and Nayak, 1996; Gonzalez *et al*., 1996; Hara *et al*., 2003; Marion *et al*., 1997; Mayer *et al*., 2007; Mazur *et al*., 2008; Ohtsu *et al*., 2002; Perez and Donis, 1995; Perez and Donis, 2001; Shapira *et al*., 2009; Sugiyama *et al*., 2009) have provided valuable data on the influenza protein-protein interactions listed in Table 3.

Severe Acute Respiratory Syndrome (SARS)

Severe acute respiratory syndrome (SARS) is an infectious human respiratory disease caused by SARS Coronavirus (HCoVs). It was first discovered in a China town Guangdong where it caused havoc in an outbreak of a typical pneumonia in 2003 and quickly spread to 23 countries. The virus was identified as a noval coronavirus and was designated to the subfamily of Coronavirinaeorder Nidovirales (Drosten *et al*., 2003). The mortality rate due to the severity of the disease was reported to be ~3 to 6% at that time (Marra *et al*., 2003).The genome of HCoV, is an enveloped positive-sense, single-stranded RNA of nearly 30 kb (He *et al*., 2004). It is transcribed into two large polyproteins pp1a and pp1abwhich upon cleavage by virally encoded proteases gives non-structural proteins such as RNA-dependent RNA polymerase (Rep), an adenosine triphosphatase (ATPase) helicase (Hel) and16 other functional non-structural proteins (nsps) (Ziebuhr *et al*., 2000). The non-structural proteins then process viral replication as well as viral proteins organization. The most important structural proteins include envelope protein E, nucleocapsid protein and membrane proteins S (Spike) and M (membrane).

Identification of interacting amino acid sequences involved in protein-protein interaction are necessary for the elucidation of the SARS-CoV replication mechanism and thus execution of anti-SARS therapeutic interference.

The nucleocapsid protein of 46 kDa is a multi-functional protein, binds the viral RNA to form the helical core structure as well helping in signal transduction and viral packaging (Hiscox *et al*., 2001).Matrix membrane protein of 25 kDa is the most abundantly produced and is essential protein for assembly of both enveloped and naked viral particles (Kuo and Masters, 2002) .

In the course of virion production the nucleocapsid protein dimerizes,a process which leads to capsid formation. Studies from yeast two hybrid system suggest that the stretch of 209 amino-acids helix rich region of the C terminal is crucial for this protein-protein interaction (Surjit *et al*., 2004).

It has been established that SARS-CoVnucleocapsid (N) and the membrane (M) proteins interact. Mammalian two hybrid system reveals that amino acid residues from 168-208 in the N protein were found to be important in the maintenance of precise conformation of the protein along with its M protein interaction (He *et al*., 2004).

The envelope protein E of the SARS-CoV is a small membrane protein of 76 amino acids. Its role in viral pathogenesis was initially unknown but it is now reported to be a virulence factor (DeDiego *et al*., 2008). Envelope

Table 3. List of influenza A virus protein-protein interactions.

	Viral Protein 1			Viral Protein 2		
S#	**Virus Type**	**NCBI Reference Sequence**	**Protein**	**Virus Type**	**NCBI Reference Sequence**	**Protein**
1.	H3N2	ABB04933	nonstructural protein 1	H3N2	ABB04936	polymerase PB1
2.	H3N2	ABB04935	polymerase PA	H3N2	ABB04936	polymerase PB1
3.	H3N2	ABB04936	polymerase PB1	H3N2	ABB04938	polymerase PB2
4.	H3N2	ABB04933	nonstructural protein 1	H3N2	ABB04938	polymerase PB2
5.	H1N1	NP_040978	matrix protein 1	H1N1	NP_040986	polymerase PA
6.	H1N1	NP_040985	polymerase 1	H1N1	NP_040986	polymerase PA
7.	H1N1	NP_040985	polymerase 1	H1N1	NP_040987	PB2 protein
8.	H1N1	NP_040980	haemagglutinin	H1N1	NP_040986	polymerase PA
9.	H1N1	NP_040980	haemagglutinin	H1N1	NP_040985	polymerase 1
10.	H1N1	NP_040980	haemagglutinin	H1N1	NP_040982	nucleocapsid protein
11.	H1N1	NP_040978	matrix protein 1	H1N1	NP_040982	nucleocapsid protein
12.	H1N1	NP_040978	matrix protein 1	H1N1	NP_040981	neuraminidase
13.	H1N1	NP_040978	matrix protein 1	H1N1	NP_040978	matrix protein 1
14.	H1N1	NP_040978	matrix protein 1	H1N1	NP_040983	nonstructural protein NS2
15.	H1N1	NP_040981	neuraminidase	H1N1	NP_040985	polymerase 1
16.	H1N1	NP_040982	nucleocapsid protein	H1N1	NP_040987	PB2 protein
17.	H1N1	NP_040982	nucleocapsid protein	H1N1	NP_040982	nucleocapsid protein
18.	H1N1	NP_040984	nonstructural protein NS1	H1N1	NP_040987	PB2 protein
19.	H1N1	NP_040984	nonstructural protein NS1	H1N1	NP_040984	nonstructural protein NS1
20.	H1N1	NP_040984	nonstructural protein NS1	H1N1	NP_040986	polymerase PA
21.	H1N1	NP_040983	nonstructural protein NS2	H1N1	NP_040984	nonstructural protein NS1
22.	H1N1	NP_040984	nonstructural protein NS1	H1N1	NP_040985	polymerase 1
23.	H1N1	NP_040981	neuraminidase	H1N1	NP_040984	nonstructural protein NS1
24.	H1N1	NP_040982	nucleocapsidprotein	H1N1	NP_040984	nonstructuralprotein NS1
25.	H1N1	NP_040980	haemagglutinin	H1N1	NP_040984	nonstructural protein NS1
26.	H1N1	NP_040978	matrix protein 1	H1N1	NP_040984	nonstructural protein NS1
27.	H1N1	NP_040983	nonstructural protein NS2	H1N1	NP_040987	PB2 protein
28.	H1N1	NP_040982	nucleocapsid protein	H1N1	NP_040986	polymerase PA

29.	H1N1	NP_040986	polymerase PA	H1N1	NP_040987	PB2 protein
30.	H1N1	NP_040986	polymerase PA	H1N1	NP_040986	polymerase PA
31.	H1N1	NP_040981	neuraminidase	H1N1	NP_040986	polymerase PA
32.	H1N1	NP_040983	nonstructural protein NS2	H1N1	NP_040986	polymerase PA
33.	H1N1	NP_040978	matrix protein 1	H1N1	NP_040985	polymerase 1
34.	H1N1	NP_040985	polymerase 1	H1N1	NP_040985	polymerase 1
35.	H1N1	NP_040983	nonstructural protein NS2	H1N1	NP_040985	polymerase 1
36.	H1N1	NP_040982	nucleocapsid protein	H1N1	NP_040985	polymerase 1
37.	H1N1	NP_040987	PB2 protein	H1N1	NP_040987	PB2 protein
38.	H1N1	ABF47959	nucleocapsid protein	H1N1	ABF47963	polymerase PB1
39.	H1N1	ABF47962	polymerase PA	H1N1	ABF47963	polymerase PB1
40.	H1N1	ABF47963	polymerase PB1	H1N1	ABF47964	PB1-F2 protein

protein is ubiquinated by the NS3 protein which is involved in the replicase complex of virus and this binding is mediated by the N-terminal ubiquitin-like domain-1 of NS3. This interaction was proved using a system of recombinant SARS-CoV expressing tagged E protein (Álvarez *et al*., 2010). Ubiquination of the E protein plays a crucial role in the multistep life cycle of the virus especially for viral entry into host cell as its inhibition leads not only to impaired entry but also RNA synthesis. (Raaben *et al*., 2010)

It is evident from above studies that the assembly of SARS-CoV is a multistep process and it involves protein-RNA and protein-protein interactions. Identification of residues involved in this type of interactions can prove to be pivotal as potential drug targets to inflect the SARS-CoV infections.

SARS coronavirus 3C like proteinase is crucial for the maturity of the viral particle. Dimerization of the proteinase is important for its activity. The dimerization reaction may be substrate–induced that renders the proteinase active (Li *et al*., 2010). The monomeric form of SARS coronavirus 3C like proteinase is not proteolytically active (Okamoto *et al*., 2010). The residue most important for SARS coronavirus dimerization is Asn28 (Barrila *et al*., 2010). Complete inactivation of the enzyme takes place by the mutation of Asn28 to alanine. Dimerization is driven by the key amino acid residues ensuring long range interaction in spite of direct interaction at the dimerization interface. The key residues involved are Ser147, Ser144 and Cys117. An extensive network of hydrogen bonds surrounds the active site of the protein. The interaction between Ser147 and Ser144 is important in the correct positioning of Met6. Met6 plays a vital role in SARS coronavirus dimerization (Barrila *et al*., 2010). The key residue Gly-11 has also been reported to be crucial for dimerization. Mutation in Gly-11 results in disruption of SARS coronavirus dimerization. The disruption results by the shortening of the alpha-helix of domain1. Moreover, the N-terminal finger cannot hold tight into the pocket of another monomer due to its dis-orientation (Chen *et al*., 2008). SARS coronavirus 3C proteinase cleaves at conserved proteolytic cleavage site containing residues GLn85 and Leu64 of NS7. These residues are crucial for the proteolytic cleavage by the 3C proteinase (Peti *et al*., 2005).

NS7 and NS8 belong to larger hetero-multimers(Su *et al*., 2006). NS7 and NS8 interact and form a hexadecamer. This resulting haxadecamer super complex can bind dsRNA. The property of binding dsRNA is conferred to the hexadecamer by the formation of a central channel. This central channel has properties that favour electrostatic interaction with nucleic acid (Zhai *et al*., 2005).

SARS coronavirus NS8 interacts with itself forming a multimeric complex (Su *et al*., 2006). NS9 has been reported to co-localize with the nucleocapsid protein and the 3C like proteinase along with NS7 and NS8 in the late endosome. The late endosomes are the sites for SARS coronavirus genome replication (Miknis *et al*., 2009).

(Imbert *et al*., 2008; Kumar *et al*., 2007; Luo *et al*., 2005; Pan *et al*., 2008; Surjit *et al*., 2004; von Brunn *et al*., 2007) have studied genome wide protein-protein interactions of SARS corona virus listed in Table 4.

Conclusion

There are many biochemical and physical assays which are in use for the determination of protein-protein interactions e.g. immunoprecipitation, protein affinity chromatography,

Table 4. List of SARS corona virus Protein-Protein interaction.

	Viral Protein 1		Viral Protein 2	
S#	**NCBI Reference Sequence**	**Protein**	**NCBI Reference Sequence**	**Protein**
1	NP_828855	matrix protein	NP_828858	nucleocapsid protein
2	NP_828856	hypothetical protein sars6	NP_828866	nsp8-pp1a/pp1ab
3	NP_828858	nucleocapsid protein	NP_828858	nucleocapsid protein
4	NP_828866	nsp8-pp1a/pp1ab	NP_828870	nsp13-pp1ab (ZD, NTPase/HEL
5	NP_828869	rna-dependent rna polymerase	NP_828870	nsp13-pp1ab (ZD, NTPase/HEL
6	NP_828870	nsp13-pp1ab (zd, ntpase/hel	NP_828870	nsp13-pp1ab (ZD, NTPase/HEL
7	NP_828859	hypothetical protein sars9b	NP_828870	nsp13-pp1ab (ZD, NTPase/HEL
8	NP_828866	nsp8-pp1a/pp1ab	NP_828871	3-to-5 exonuclease
9	NP_828859	hypothetical protein sars9b	NP_828871	3-to-5 exonuclease
10	NP_828859	hypothetical protein sars9b	NP_828872	endoRNAse
11	NP_828854	protein e	NP_828860	leader protein
12	NP_828854	protein e	NP_828866	nsp8-pp1a/pp1ab
13	NP_828854	protein e	NP_904321	nsp11-pp1a
14	NP_828854	protein e	NP_828854	protein E
15	NP_828852	hypothetical protein sars3a	NP_828860	leader protein
16	NP_828861	counterpart of mhv p65	NP_828861	counterpart of MHV p65
17	NP_828861	counterpart of mhv p65	NP_904322	nsp4-pp1a/pp1ab
18	NP_828861	counterpart of mhv p65	NP_828864	nsp6-pp1a/pp1ab (TM3)
19	NP_828861	counterpart of mhv p65	NP_828866	nsp8-pp1a/pp1ab
20	NP_828861	counterpart of mhv p65	NP_904321	nsp11-pp1a
21	NP_828861	counterpart of mhv p65	NP_828873	2-O-ribose methyltransferase (2-o-MT)
22	NP_828852	hypothetical protein sars3a	NP_828861	counterpart of MHV p65
23	NP_828861	counterpart of mhv p65	NP_828862	nsp3-pp1a/pp1ab
24	NP_828852	hypothetical protein sars3a	NP_828862	nsp3-pp1a/pp1ab
25	NP_828859	hypothetical protein sars9b	NP_828862	nsp3-pp1a/pp1ab
26	NP_828863	3c-like proteinase	NP_828863	3C-like proteinase
27	NP_828863	3c-like proteinase	NP_828865	nsp7-pp1a/pp1ab
28	NP_828863	3c-like proteinase	NP_828866	nsp8-pp1a/pp1ab
29	NP_828859	hypothetical protein sars9b	NP_828863	3C-like proteinase

30	NP_828864	nsp6-pp1a/pp1ab (tm3)	NP_828866	nsp8-pp1a/pp1ab
31	NP_828865	nsp7-pp1a/pp1ab	NP_828865	nsp7-pp1a/pp1ab
32	NP_828865	nsp7-pp1a/pp1ab	NP_828866	nsp8-pp1a/pp1ab
33	NP_828859	hypothetical protein sars9b	NP_828865	nsp7-pp1a/pp1ab
34	NP_828866	nsp8-pp1a/pp1ab	NP_828866	nsp8-pp1a/pp1ab
35	NP_828859	hypothetical protein sars9b	NP_828866	nsp8-pp1a/pp1ab
36	NP_828864	nsp6-pp1a/pp1ab (tm3)	NP_828867	nsp9-pp1a/pp1ab
37	NP_828865	nsp7-pp1a/pp1ab	NP_828867	nsp9-pp1a/pp1ab
38	NP_828866	nsp8-pp1a/pp1ab	NP_828867	nsp9-pp1a/pp1ab
39	NP_828852	hypothetical protein sars3a	NP_828868	formerly known as growth-factor-like protein (GFL)
40	NP_828859	hypothetical protein sars9b	NP_828868	formerly known as growth-factor-like protein (GFL)
41	NP_828866	nsp8-pp1a/pp1ab	NP_828869	RNA-dependent RNA polymerase
42	NP_828852	hypothetical protein sars3a	NP_828869	RNA-dependent RNA polymerase
43	NP_828859	hypothetical protein sars9b	NP_828869	RNA-dependent RNA polymerase
44	NP_828865	nsp7-pp1a/pp1ab	NP_828870	nsp13-pp1ab (ZD, NTPase/HEL
45	NP_828853	hypothetical protein sars3b	NP_828854	protein E
46	NP_828854	protein e	NP_849175	hypothetical protein sars7b
47	NP_828854	protein e	NP_828859	hypothetical protein sars9b
48	NP_828852	hypothetical protein sars3a	NP_828855	matrix protein
49	NP_828852	hypothetical protein sars3a	NP_828858	nucleocapsid protein
50	NP_828856	hypothetical protein sars6	NP_828862	nsp3-pp1a/pp1ab
51	NP_828856	hypothetical protein sars6	NP_849175	hypothetical protein sars7b
52	NP_828857	hypothetical protein sars7a	NP_828862	nsp3-pp1a/pp1ab
53	NP_828851	e2 glycoprotein precursor	NP_828857	hypothetical protein sars7a
54	NP_849176	hypothetical protein sars8a	NP_828866	nsp8-pp1a/pp1ab
55	NP_849176	hypothetical protein sars8a	NP_828872	endoRNAse
56	NP_849176	hypothetical protein sars8a	NP_849177	hypothetical protein sars8b
57	NP_828859	hypothetical protein sars9b	NP_849176	hypothetical protein sars8a
58	NP_849177	hypothetical protein sars8b	NP_828862	nsp3-pp1a/pp1ab
59	NP_849177	hypothetical protein sars8b	NP_828866	nsp8-pp1a/pp1ab

60	NP_828851	e2 glycoprotein precursor	NP_849177	hypothetical protein sars8b
61	NP_849175	hypothetical protein sars7b	NP_849177	hypothetical protein sars8b
62	NP_828859	hypothetical protein sars9b	NP_849177	hypothetical protein sars8b
63	NP_828859	hypothetical protein sars9b	NP_849175	hypothetical protein sars7b
64	NP_828859	hypothetical protein sars9b	NP_828859	hypothetical protein sars9b
65	NP_828862	nsp3-pp1a/pp1ab	NP_828862	nsp3-pp1a/pp1ab
66	NP_828862	nsp3-pp1a/pp1ab	NP_904322	nsp4-pp1a/pp1ab
67	NP_828862	nsp3-pp1a/pp1ab	NP_828869	RNA-dependent RNA polymerase
68	NP_828862	nsp3-pp1a/pp1ab	NP_828866	nsp8-pp1a/pp1ab
69	NP_828861	counterpart of mhv p65	NP_828865	nsp7-pp1a/pp1ab
70	NP_828853	hypothetical protein sars3b	NP_828866	nsp8-pp1a/pp1ab
71	NP_849175	hypothetical protein sars7b	NP_828867	nsp9-pp1a/pp1ab
72	NP_828868	formerly known as growth-factor-like protein (gfl)	NP_828871	3-to-5 exonuclease
73	NP_828868	formerly known as growth-factor-like protein (gfl)	NP_828873	2-O-ribose methyltransferase (2-o-MT)
74	NP_828863	3c-like proteinase	NP_828869	RNA-dependent RNA polymerase
75	NP_828853	hypothetical protein sars3b	NP_828869	RNA-dependent RNA polymerase
76	NP_828853	hypothetical protein sars3b	NP_828870	nsp13-pp1ab (ZD, NTPase/HEL
77	NP_828863	3c-like proteinase	NP_828871	3-to-5 exonuclease
78	NP_828853	hypothetical protein sars3b	NP_828871	3-to-5 exonuclease
79	NP_828861	counterpart of mhv p65	NP_828872	endoRNAse
80	NP_828872	endornase	NP_828872	endoRNAse
81	NP_849175	hypothetical protein sars7b	NP_828873	2-O-ribose methyltransferase (2-o-MT)
82	NP_828858	nucleocapsid protein	NP_828873	2-O-ribose methyltransferase (2-o-MT)
83	NP_828852	hypothetical protein sars3a	NP_849177	hypothetical protein sars8b
84	NP_828854	protein e	NP_828857	hypothetical protein sars7a
85	NP_828855	matrix protein	NP_828857	hypothetical protein sars7a
86	NP_828855	matrix protein	NP_849177	hypothetical protein sars8b
87	NP_849177	hypothetical protein sars8b	NP_828867	nsp9-pp1a/pp1ab
88	NP_828858	nucleocapsid protein	NP_849177	hypothetical protein sars8b
89	NP_828862	nsp3-pp1a/pp1ab	NP_828863	3C-like proteinase
90	NP_828862	nsp3-pp1a/pp1ab	NP_828870	nsp13-pp1ab (ZD,

				NTPase/HEL
91	NP_828862	nsp3-pp1a/pp1ab	NP_828871	3-to-5 exonuclease
92	NP_828862	nsp3-pp1a/pp1ab	NP_828864	nsp6-pp1a/pp1ab (TM3)
93	NP_828865	nsp7-pp1a/pp1ab	NP_828869	RNA-dependent RNA polymerase
94	NP_828865	nsp7-pp1a/pp1ab	NP_828871	3-to-5 exonuclease
95	NP_828866	nsp8-pp1a/pp1ab	NP_828872	endoRNAse
96	NP_828860	leader protein	NP_828867	nsp9-pp1a/pp1ab
97	NP_828867	nsp9-pp1a/pp1ab	NP_828871	3-to-5 exonuclease
98	NP_828863	3c-like proteinase	NP_828868	formerly known as growth-factor-like protein (GFL)
99	NP_828862	nsp3-pp1a/pp1ab	NP_828873	2-O-ribose methyltransferase (2-o-MT)
100	NP_828861	counterpart of mhv p65	NP_828869	RNA-dependent RNA polymerase
101	NP_828869	rna-dependent rna polymerase	NP_828871	3-to-5 exonuclease
102	NP_828869	rna-dependent rna polymerase	NP_828872	endoRNAse
103	NP_828869	rna-dependent rna polymerase	NP_828873	2-O-ribose methyltransferase (2-o-MT)
104	NP_828866	nsp8-pp1a/pp1ab	NP_828868	formerly known as growth-factor-like protein (GFL)
105	NP_828866	nsp8-pp1a/pp1ab	NP_828873	2-O-ribose methyltransferase (2-o-MT)
106	NP_828862	nsp3-pp1a/pp1ab	NP_828865	nsp7-pp1a/pp1ab
107	NP_828862	nsp3-pp1a/pp1ab	NP_828872	endoRNAse
108	NP_828861	counterpart of mhv p65	NP_828867	nsp9-pp1a/pp1ab
109	NP_828862	nsp3-pp1a/pp1ab	NP_828867	nsp9-pp1a/pp1ab
110	NP_828863	3c-like proteinase	NP_828867	nsp9-pp1a/pp1ab
111	NP_828867	nsp9-pp1a/pp1ab	NP_828868	formerly known as growth-factor-like protein (GFL)
112	NP_828867	nsp9-pp1a/pp1ab	NP_828869	RNA-dependent RNA polymerase
113	NP_828867	nsp9-pp1a/pp1ab	NP_828870	nsp13-pp1ab (ZD, NTPase/HEL
114	NP_828867	nsp9-pp1a/pp1ab	NP_828872	endoRNAse

affinity blotting and western blot experiments. Recent advances in high-throughput methods for the detection of protein-protein interaction, such as yeast two-hybrid and mass spectrometry techniques, have led to a rapid expansion of such data for a wide range of organisms. In such a way, several studies address the viral-viral and viral-host protein interactions depicting the molecular mechanism involved in the pathogenicity of viruses. Most of the pathogenicity occurs due to the interaction of host and viral molecular factors which ultimately affect normal cellular signaling pathways. But the first line of viral action is viral protein-protein interactions which on the second hand initiate their pathogenesis. Anti-viral therapies based on blocking viral-host interactions come with certain discrepancies such as disturbances of the normal cellular processes. New treatment options specific in targeting viral protein-protein interaction can be proved fruitful. At this time, new classes of inhibitors are needed which specifically hinder only the post modifications of viral proteins by other virus encoded proteins. This treatment strategy will definitely prove to be a better option with safe hands on host molecular identities.

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REFERENCES

1. Ahmed N and HE Heslop. 2006. Viral lymphomagenesis. Current Opinion in Hematology 13 (4): 254.

2. Álvarez E, ML DeDiego, JL Nieto-Torres, JM Jiménez-Guardeño, L Marcos-Villar and L Enjuanes. 2010. The envelope protein of severe acute respiratory syndrome coronavirus interacts with the non-structural protein 3 and is ubiquitinated. Virology 402 (2): 281-291.
3. Area E, J Martín-Benito, P Gastaminza, E Torreira, JM Valpuesta, JL Carrascosa and J Ortín. 2004. 3D structure of the influenza virus polymerase complex: localization of subunit domains. Proceedings of the National Academy of Sciences of the United States of America 101 (1): 308.
4. Arias CF, F Preugschat and JH Strauss. 1993. Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology 193 (2): 888-899.
5. Barrila J, SB Gabelli, U Bacha, LM Amzel and E Freire. 2010. Mutation of Asn28 Disrupts the Dimerization and Enzymatic Activity of SARS 3CLpro. Biochemistry 49 (20): 4308-4317.
6. Barth H, TJ Liang and TF Baumert. 2006. Hepatitis C virus entry: molecular biology and clinical implications. Hepatology 44 (3): 527-535.
7. Bartosch B, J Dubuisson and FL Cosset. 2003. Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. The Journal of experimental medicine 197 (5): 633.
8. Biswas SK, PL Boutz and DP Nayak. 1998. Influenza virus nucleoprotein interacts with influenza virus polymerase proteins. Journal of virology 72 (7): 5493-5501.
9. Biswas SK and DP Nayak. 1996. Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. Journal of virology 70 (10): 6716-6722.
10. Brazzoli M, A Helenius, SKH Foung, M Houghton, S Abrignani and M Merola. 2005. Folding and dimerization of hepatitis C virus E1 and E2 glycoproteins in stably transfected CHO cells. Virology 332 (1): 438-453.
11. Brooks AJ, M Johansson, AV John, Y Xu, DA Jans and SG Vasudevan. 2002. The interdomain region of dengue NS5 protein that binds to the viral helicase NS3 contains independently functional importin beta 1 and importin alpha/beta-recognized nuclear localization signals. Journal of Biological Chemistry 277 (39): 36399-36407.
12. Carrere-Kremer S, C Montpellier-Pala, L Cocquerel, C Wychowski, F Penin and J Dubuisson. 2002. Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. Journal of virology 76 (8): 3720-3730.
13. Chen S, T Hu, J Zhang, J Chen, K Chen, J Ding, H Jiang and X Shen. 2008. Mutation of Gly-11 on the Dimer Interface Results in the Complete Crystallographic Dimer Dissociation of Severe Acute Respiratory Syndrome Coronavirus 3C-like Protease. Journal of Biological Chemistry 283 (1): 554.
14. Choo Q, A Weiner, L Overby, G Kuo, M Houghton and D Bradley. 1990. Hepatitis C virus: the major causative agent of viral non-A, non-B hepatitis. British medical bulletin 46 (2): 423.
15. Clum S, KE Ebner and R Padmanabhan. 1997. Cotranslational membrane insertion of the serine proteinase precursor NS2B-NS3(Pro) of dengue virus type 2 is required for efficient in vitro processing and is mediated through the hydrophobic regions of NS2B. Journal of Biological Chemistry 272 (49): 30715-30723.
16. Courageot MP, MP Frenkiel, CD Dos Santos, V Deubel and P Despres. 2000. Alpha-glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. Journal of virology 74 (1): 564-572.
17. DeDiego ML, L Pewe, E Alvarez, MT Rejas, S Perlman and L Enjuanes. 2008. Pathogenicity of severe acute respiratory coronavirus deletion mutants in hACE-2 transgenic mice. Virology 376 (2): 379-389.
18. Dimitrova M, I Imbert, MP Kieny and C Schuster. 2003. Protein-protein interactions between hepatitis C virus nonstructural proteins. Journal of virology 77 (9): 5401-5414.
19. Drosten C, S Günther, W Preiser, S Van Der Werf, HR Brodt, S Becker, H Rabenau, M Panning, L Kolesnikova and RAM Fouchier. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. The New England journal of medicine 348 (20): 1967-1976.
20. Drummer HE and P Poumbourios. 2004. Hepatitis C virus glycoprotein E2 contains a membrane-proximal heptad repeat sequence that is essential for E1E2 glycoprotein heterodimerization and viral entry. Journal of Biological Chemistry 279 (29): 30066-30072.
21. Dubuisson J, HH Hsu, RC Cheung, HB Greenberg, DG Russell and CM Rice. 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. Journal of virology 68 (10): 6147-6160.
22. Flajolet M, G Rotondo, L Daviet, F Bergametti, G Inchauspe, P Tiollais, C Transy and P Legrain. 2000. A genomic approach of the hepatitis C virus generates a protein interaction map. Gene 242 (1-2): 369-379.
23. Fodor E and M Smith. 2004. The PA subunit is required for efficient nuclear accumulation of the PB1 subunit of the influenza A virus RNA polymerase complex. Journal of virology 78 (17): 9144-9153.
24. Fouchier RA, V Munster, A Wallensten, TM Bestebroer, S Herfst, D Smith, GF Rimmelzwaan, B Olsen and AD Osterhaus. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. Journal of virology 79 (5): 2814-2822.
25. Fridell RA, D Qiu, L Valera, C Wang, RE Rose and M Gao. 2011. Distinct Functions of NS5A in HCV RNA Replication Uncovered by Studies with the NS5A Inhibitor BMS-790052. Journal of virology: JVI. 00253-00211v00251.

26. Ghedin E, NA Sengamalay, M Shumway, J Zaborsky, T Feldblyum, V Subbu, DJ Spiro, J Sitz, H Koo and P Bolotov. 2005. Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. Nature 437 (7062): 1162-1166.
27. Goh PY, YJ Tan, SP Lim, SG Lim, YH Tan and WJ Hong. 2001. The hepatitis C virus core protein interacts with NS5A and activates its caspase-mediated proteolytic cleavage. Virology 290 (2): 224-236.
28. Gonzalez S, T Zurcher and J Ortin. 1996. Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. Nucleic Acids Research 24 (22): 4456-4463.
29. Hara K, M Shiota, H Kido, K Watanabe, K Nagata and T Toyoda. 2003. Inhibition of the protease activity of influenza virus RNA polymerase PA subunit by viral matrix protein. Microbiology and Immunology 47 (7): 521-526.
30. He R, A Leeson, M Ballantine, A Andonov, L Baker, F Dobie, Y Li, N Bastien, H Feldmann, U Strocher, S Theriault, T Cutts, J Cao, TF Booth, FA Plummer, S Tyler and X Li. 2004. Characterization of protein-protein interactions between the nucleocapsid protein and membrane protein of the SARS coronavirus. Virus Research 105 (2): 121-125.
31. Hebner CM and LA Laimins. 2006. Human papillomaviruses: basic mechanisms of pathogenesis and oncogenicity. Reviews in medical virology 16 (2): 83-97.
32. Hiscox JA, T Wurm, L Wilson, P Britton, D Cavanagh and G Brooks. 2001. The coronavirus infectious bronchitis virus nucleoprotein localizes to the nucleolus. Journal of Virology 75 (1): 506.
33. Imbert I, EJ Snijder, M Dimitrova, JC Guillemot, P Lecine and B Canard. 2008. The SARS-Coronavirus PLnc domain of nsp3 as a replication/transcription scaffolding protein. Virus Research 133 (2): 136-148.
34. Ishido S, T Fujita and H Hotta. 1998. Complex Formation of NS5B with NS3 and NS4A Proteins of Hepatitis C Virus* 1. Biochemical and biophysical research communications 244 (1): 35-40.
35. Johansson M, AJ Brooks, DA Jans and SG Vasudevan. 2001. A small region of the dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-beta and the viral helicase, NS3. Journal of general virology 82 (Pt 4): 735-745.
36. Jones CT, L Ma, JW Burgner, TD Groesch, CB Post and RJ Kuhn. 2003. Flavivirus capsid is a dimeric alpha-helical protein. Journal of virology 77 (12): 7143-7149.
37. Khu YL, E Koh, SP Lim, YH Tan, S Brenner, SG Lim, WJ Hong and PY Goh. 2001. Mutations that affect dimer formation and helicase activity of the hepatitis C virus helicase. Journal of virology 75 (1): 205-214.
38. Kuhn RJ and MG Rossmann. 1995. Virology. When it's better to lie low. Nature 375 (6529): 275-276.
39. Kuhn RJ, W Zhang, MG Rossmann, SV Pletnev, J Corver, E Lenches, CT Jones, S Mukhopadhyay, PR Chipman, EG Strauss, TS Baker and JH Strauss. 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 108 (5): 717-725.
40. Kumar P, V Gunalan, B Liu, VTK Chow, J Druce, C Birch, M Catton, BC Fielding, YJ Tan and SK Lal. 2007. The nonstructural protein 8 (nsp8) of the SARS coronavirus interacts with its ORF6 accessory protein. Virology 366 (2): 293-303.
41. Kummerer BM and CM Rice. 2002. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. Journal of Virology 76 (10): 4773-84.
42. Kuo L and PS Masters. 2002. Genetic evidence for a structural interaction between the carboxy termini of the membrane and nucleocapsid proteins of mouse hepatitis virus. Journal of Virology 76 (10): 4987.
43. Lanford RE, L Notvall, D Chavez, R White, G Frenzel, C Simonsen and J Kim. 1993. Analysis of hepatitis C virus capsid, E1, and E2/NS1 proteins expressed in insect cells. Virology 197 (1): 225-235.
44. Leung D, K Schroder, H White, NX Fang, MJ Stoermer, G Abbenante, JL Martin, PR Young and DP Fairlie. 2001. Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors. Journal of Biological Chemistry 276 (49): 45762-45771.
45. Li C, Y Qi, X Teng, Z Yang, P Wei, C Zhang, L Tan, L Zhou, Y Liu and L Lai. 2010. Maturation Mechanism of Severe Acute Respiratory Syndrome (SARS) Coronavirus 3C-like Proteinase. Journal of Biological Chemistry 285 (36): 28134.
46. Lim SG, YJ Tan, PY Goh, SP Lim and WJ Hong. 2006. Use of an in vitro model and yeast two-hybrid system to investigate the pathogenesis of hepatitis C. Intervirology 49 (1-2): 44-50.
47. Lin C, JW Wu, K Hsiao and MS Su. 1997. The hepatitis C virus NS4A protein: interactions with the NS4B and NS5A proteins. Journal of Virology 71 (9): 6465-6471.
48. Lindenbach BD and CM Rice. 1999. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. Journal of virology 73 (6): 4611-4621.
49. Liu WJ, HB Chen and AA Khromykh. 2003. Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. Journal of Virology 77 (14): 7804.
50. Lo S, MJ Selby and J Ou. 1996. Interaction between hepatitis C virus core protein and E1 envelope protein. Journal of Virology 70 (8): 5177.
51. Lohmann V, J Koch and R Bartenschlager. 1996. Processing pathways of the hepatitis C virus proteins. Journal of hepatology 24 (2 Suppl): 11.
52. Love RA, O Brodsky, MJ Hickey, PA Wells and CN Cronin. 2009. Crystal structure of a novel dimeric

form of NS5A domain I protein from hepatitis C virus. Journal of virology 83 (9): 4395.
53. Luo H, F Ye, K Chen, X Shen and H Jiang. 2005. SR-rich motif plays a pivotal role in recombinant SARS coronavirus nucleocapsid protein multimerization. Biochemistry 44 (46): 15351-15358.
54. Ma HC, CH Ke, TY Hsieh and SY Lo. 2002. The first hydrophobic domain of the hepatitis C virus E1 protein is important for interaction with the capsid protein. Journal of general virology 83 (Pt 12): 3085-3092.
55. Ma L, CT Jones, TD Groesch, RJ Kuhn and CB Post. 2004. Solution structure of dengue virus capsid protein reveals another fold. Proceedings of the National Academy of Sciences U S A 101 (10): 3414-3419.
56. Marion RM, T Zurcher, S de la Luna and J Ortin. 1997. Influenza virus NS1 protein interacts with viral transcription-replication complexes in vivo. Journal of general virology 78 (Pt 10): 2447-2451.
57. Marra MA, SJM Jones, CR Astell, RA Holt, A Brooks-Wilson, YSN Butterfield, J Khattra, JK Asano, SA Barber and SY Chan. 2003. The genome sequence of the SARS-associated coronavirus. Science 300 (5624): 1399.
58. Matsumoto M, SB Hwang, KS Jeng, N Zhu and MM Lai. 1996. Homotypic interaction and multimerization of hepatitis C virus core protein. Virology 218 (1): 43-51.
59. Mayer D, K Molawi, L Martinez-Sobrido, A Ghanem, S Thomas, S Baginsky, J Grossmann, A Garcia-Sastre and M Schwemmle. 2007. Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches. Journal of Proteome Research 6 (2): 672-682.
60. Mazur I, D Anhlan, D Mitzner, L Wixler, U Schubert and S Ludwig. 2008. The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cell Microbiol 10 (5): 1140-1152.
61. Miknis ZJ, EF Donaldson, TC Umland, RA Rimmer, RS Baric and LW Schultz. 2009. Severe acute respiratory syndrome coronavirus nsp9 dimerization is essential for efficient viral growth. Journal of virology 83 (7): 3007.
62. Molenkamp R, EA Kooi, MA Lucassen, S Greve, JC Thijssen, WJ Spaan and PJ Bredenbeek. 2003. Yellow fever virus replicons as an expression system for hepatitis C virus structural proteins. Journal of virology 77 (2): 1644-1648.
63. Mousseau G, S Kota, V Takahashi, D Frick and AD Strosberg. 2011. Dimerization-driven interaction of hepatitis C virus core protein with NS3 helicase. Journal of general virology 92 (1): 101.
64. Nielsen SU, MF Bassendine, AD Burt, DJ Bevitt and GL Toms. 2004. Characterization of the genome and structural proteins of hepatitis C virus resolved from infected human liver. Journal of general virology 85 (6): 1497.
65. Niyomrattanakit P, P Winoyanuwattikun, S Chanprapaph, C Angsuthanasombat, S Panyim and G Katzenmeier. 2004. Identification of residues in the dengue virus type 2 NS2B cofactor that are critical for NS3 protease activation. Journal of virology 78 (24): 13708-13716.
66. Nolandt O, V Kern, H Muller, E Pfaff, L Theilmann, R Welker and HG Krausslich. 1997. Analysis of hepatitis C virus core protein interaction domains. Journal of general virology 78 (Pt 6): 1331-1340.
67. Ohtsu Y, Y Honda, Y Sakata, H Kato and T Toyoda. 2002. Fine mapping of the subunit binding sites of influenza virus RNA polymerase. Microbiology and Immunology 46 (3): 167-175.
68. Okamoto DN, LCG Oliveira, MY Kondo, MHS Cezari, Z Szeltner, T Juhász, MA Juliano, L Polgár, L Juliano and IE Gouvea. 2010. Increase of SARS-CoV 3CL peptidase activity due to macromolecular crowding effects in the milieu composition. Biological Chemistry 391 (12): 1461-1468.
69. Op De Beeck A, R Montserret, S Duvet, L Cocquerel, R Cacan, B Barberot, M Le Maire, F Penin and J Dubuisson. 2000. The transmembrane domains of hepatitis C virus envelope glycoproteins E1 and E2 play a major role in heterodimerization. Journal of Biological Chemistry 275 (40): 31428-31437.
70. Pan J, X Peng, Y Gao, Z Li, X Lu, Y Chen, M Ishaq, D Liu, ML Dediego, L Enjuanes and D Guo. 2008. Genome-wide analysis of protein-protein interactions and involvement of viral proteins in SARS-CoV replication. PLoS One 3 (10): e3299.
71. Perez DR and RO Donis. 1995. A 48-amino-acid region of influenza A virus PB1 protein is sufficient for complex formation with PA. Journal of virology 69 (11): 6932-6939.
72. Perez DR and RO Donis. 2001. Functional analysis of PA binding by influenza a virus PB1: effects on polymerase activity and viral infectivity. Journal of virology 75 (17): 8127-8136.
73. Peti W, MA Johnson, T Herrmann, BW Neuman, MJ Buchmeier, M Nelson, J Joseph, R Page, RC Stevens and P Kuhn. 2005. Structural genomics of the severe acute respiratory syndrome coronavirus: nuclear magnetic resonance structure of the protein nsP7. Journal of virology 79 (20): 12905.
74. Piccininni S, A Varaklioti, M Nardelli, B Dave, KD Raney and JE McCarthy. 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. Journal of Biological Chemistry 277 (47): 45670-45679.
75. Preugschat F and JH Strauss. 1991. Processing of nonstructural proteins NS4A and NS4B of dengue 2 virus in vitro and in vivo. Virology 185 (2): 689-697.
76. Pryor MJ, L Azzola, PJ Wright and AD Davidson. 2004. Histidine 39 in the dengue virus type 2 M protein has an important role in virus assembly. Journal of general virology 85 (Pt 12): 3627-3636.
77. Pryor MJ and PJ Wright. 1994. Glycosylation mutants of dengue virus NS1 protein. Journal of general virology 75 (Pt 5): 1183-1187.
78. Raaben M, CC Posthuma, MH Verheije, EG te Lintelo, M Kikkert, JW Drijfhout, EJ Snijder, PJM

Rottier and CAM de Haan. 2010. The ubiquitin-proteasome system plays an important role during various stages of the coronavirus infection cycle. Journal of virology 84 (15): 7869.
79. Rey FA, FX Heinz, C Mandl, C Kunz and SC Harrison. 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 375 (6529): 291-298.
80. Rice C. 1996. Flaviviridae: the viruses and their replication. Fields virology 1: 931-959.
81. Shapira SD, I Gat-Viks, BO Shum, A Dricot, MM de Grace, L Wu, PB Gupta, T Hao, SJ Silver, DE Root, DE Hill, A Regev and N Hacohen. 2009. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell 139 (7): 1255-1267.
82. Shirota Y, H Luo, W Qin, S Kaneko, T Yamashita, K Kobayashi and S Murakami. 2002. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. Journal of Biological Chemistry 277 (13): 11149-11155.
83. Su D, Z Lou, F Sun, Y Zhai, H Yang, R Zhang, A Joachimiak, XC Zhang, M Bartlam and Z Rao. 2006. Dodecamer structure of severe acute respiratory syndrome coronavirus nonstructural protein nsp10. Journal of virology 80 (16): 7902.
84. Sugiyama K, E Obayashi, A Kawaguchi, Y Suzuki, JR Tame, K Nagata and SY Park. 2009. Structural insight into the essential PB1-PB2 subunit contact of the influenza virus RNA polymerase. EMBO J 28 (12): 1803-1811.
85. Surjit M, B Liu, P Kumar, VT Chow and SK Lal. 2004. The nucleocapsid protein of the SARS coronavirus is capable of self-association through a C-terminal 209 amino acid interaction domain. Biochemical and Biophysical Research Communications 317 (4): 1030-1036.
86. Tellinghuisen TL and CM Rice. 2002. Interaction between hepatitis C virus proteins and host cell factors. Current opinion in microbiology 5 (4): 419-427.
87. Uchida M, N Hino, T Yamanaka, H Fukushima, T Imanishi, Y Uchiyama, T Kodama and T Doi. 2002. Hepatitis C virus core protein binds to a C-terminal region of NS5B RNA polymerase. Hepatology research 22 (4): 297-306.
88. Umareddy I, A Chao, A Sampath, F Gu and SG Vasudevan. 2006. Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. Journal of general virology 87 (Pt 9): 2605-2614.
89. von Brunn A, C Teepe, JC Simpson, R Pepperkok, CC Friedel, R Zimmer, R Roberts, R Baric and J Haas. 2007. Analysis of intraviral protein-protein interactions of the SARS coronavirus ORFeome. PLoS One 2 (5): e459.
90. Wang SH, WJ Syu and ST Hu. 2004. Identification of the homotypic interaction domain of the core protein of dengue virus type 2. Journal of general virology 85 (Pt 8): 2307-2314.
91. Welsch C, M Albrecht, J Maydt, E Herrmann, MW Welker, C Sarrazin, A Scheidig, T Lengauer and S Zeuzem. 2007. Structural and functional comparison of the non-structural protein 4B in flaviviridae. Journal of Molecular Graphics and Modelling 26 (2): 546-557.
92. Yan BS, MH Tam and WJ Syu. 1998. Self-association of the C-terminal domain of the hepatitis-C virus core protein. European Journal of Biochemistry 258 (1): 100-106.
93. Yasui K, T Wakita, K Tsukiyama-Kohara, SI Funahashi, M Ichikawa, T Kajita, D Moradpour, JR Wands and M Kohara. 1998. The native form and maturation process of hepatitis C virus core protein. Journal of virology 72 (7): 6048.
94. Zhai Y, F Sun, X Li, H Pang, X Xu, M Bartlam and Z Rao. 2005. Insights into SARS-CoV transcription and replication from the structure of the nsp7–nsp8 hexadecamer. Nature Structural & Molecular Biology 12 (11): 980-986.
95. Ziebuhr J, EJ Snijder and AE Gorbalenya. 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. Journal of general virology 81 (4): 853.

Original Article

Small Interfering RNA - Modern Approach for Intervening Pathological Conditions

Shazia Choudhary, Sheeba Murad Mall, Sana Gul, Hayat Khan, Samra Khalid Summar Gul, Noureen Ehsan, Memoona Noreen
Atta-ur-Rahman School of Applied Biosciences
sheebamall@yahoo.com

Abstract

RNA interference (RNAi) refers to the inhibition of gene expression by small double- stranded RNA molecules. This technology can prove to be a breakthrough biological discovery of the decade as it has the potential to revolutionize the field of therapeutics. RNA interference (RNAi) through small interfering RNA (siRNA) is currently being evaluated for its efficacy to be used in therapeutics as well as prophylactic strategies. Many studies are being conducted across the globe to optimize the siRNA delivery systems (in terms of safe, stable and efficient delivery) in various disorders. There are a number of diseases such as autoimmune diseases, cancer associated pathological changes, bacterial and viral induced disorders, where RNAi pathway can be explored and RNAi technology can be used as a tool to intervene such abnormalities. This review is an effort to review latest advancements in the field of siRNA based therapy development and the pits and falls generally encountered in the use of this technology.

Key words: RNA interference, small interfering RNA, delivery mode.

Remarkable Contributions in Field of Therapeutics By siRNA

Small interfering RNA (siRNA) induced gene silencing is one of the latest advancements in the field of molecular biology. siRNA belongs to a class of double-stranded RNA molecules, 20-25 nucleotides in length. These small nucleotide sequences can interfere with the expression of specific gene at a post transcriptional level, therefore causing a targeted disruption of a gene (Scherr *et al*., 2003). This technology is employed in research as well as in designing the new therapeutic strategies.

Although siRNA is widely used for studying a particular gene function and for elucidating signaling pathways however, therapeutics remains to be at the forefront of siRNA based technology (Ganesan *et al*., 2008). Clinical trials of RNAi- based therapies are being conducted. Targeted disruption of the culprit genes responsible for various pathological conditions resulting either from pathogenic interventions or due to abnormal functioning of body`s own immune system i.e autoimmunity is possible through siRNA (Hokaiwado *et al,*. 2008; McNamara *et al.,* 2006). Studies are being conducted in order to evaluate the use of siRNA technique in a wide range of pathologies e.g. in cardiovascular diseases (Tang *et al.*, 2007) various infections, cancers and autoimmunity in various *in vitro* as well as in vivo models, as well as in clinical trials (Eckstein *et al.,* 2010; Kumar *et al.,* 2008; Pruijn. 2006).

Challenges Faced By the Researchers

The use of siRNA in therapeutics is a promising technology, however, there are some limitations offered by this state of art molecular tool (Shimaoka *et al.,* 2009). Two major issues generally faced by the researchers are; the stability of siRNA and the targeted delivery of siRNA to the desired cell population while maintaining a steady state environment.

Review of all the available data would be beyond the scope of this article, yet we have tried to focus on some recent, innovative advancements in the field of siRNA especially for therapeutic purposes and pits and fall related to it.

Direct administration of naked siRNA can silence the specific gene expression however due to the serum nucleases (RNA degrading enzymes), siRNA has relatively short half life (Lares *et al*., 2010; Poliseno *et al.,* 2004). In order to ensure maximum, stable and targeted incorporation of siRNA, various research groups employed various strategies. One of the developments is to couple siRNA with an immunoliposome. Zheung et al in 2009 was able to show the successful targeted delivery of siRNA to dendritic cells (DCs) (Zheng *et al.*, 2009). In an effort to generate tolerogenic dendritic cells (DCs), Zheung et al developed siRNA against CD40, a co-stimulatory molecule expressed by DCs. For this purpose they employed a polyethylene coated liposome containing the CD40 specific siRNA in order to suppress the CD40 gene expression. In order to ensure the targeted delivery of siRNA specifically to DCs they coupled the siRNA containing immunoliposomes with monoclonal antibody (NLDC-145) specifically targeting DC specific surface marker DEC-205. The final product encapsulated by an immunoliposome not only ensured the targeted delivery by means of the incorporated antibody but also protected the enclosed siRNA against RNA degrading enzymes (Zheung *et al.,*2009).

Nanoparticle (NP) technology is yet another exciting innovation which is currently being evaluated for its scientific utilization in various fields. Use of NPs in therapeutic and prophylactic purposes is also being

Corresponding Author: Sheeba Murad Mall

evaluated (Dykxhoorn *et al.,* 2006). In face of various genomic approaches being employed for the treatment of cancers, siRNA based NPs offers a very promising tool for the down regulation of genes contributing towards cancer sustenance and progression (Aigner *et al.,* 2006). One such study specifically targeted the transferrin receptors expressed by tumor cells, which are typically up regulated in cancer cells. Transferrin receptors are responsible for providing cellular iron needed for cell survival (Muñoz *et al*., 2011) and hydroxylation of hypoxia-inducible factor-1α (HIF-α) by prolyl hydroxylases (PHD). The blocking of the transferrin receptors on cancer cells can be an effective way to prevent the cancer cell growth. Davis in 2009, conducted a study in which siRNA against transferin receptors was coupled with cyclodextrin containing polymer, polyethylene glycol (PEG) were assembled together into <100nm colloidal NPs (Davis. 2009). The pre-clinical and clinical use of such linear polymers has been demonstrated (Heidel. 2006). Results have shown the cell specific delivery and efficient gene silencing. Another similar study conducted by Shyh et al in 2008 (Li *et al.,* 2008) reported the use of NPs to deliver siRNA specifically to the tumor cells. Epidermal growth factor receptor (EGFR) is a cell surface receptor for the extracellular protein ligands and is involved in initiating signaling cascades, providing the necessary signal for cell proliferation, cell migration and cell adhesion (Koff *et al.,* 2008; Garrido *et al.,* 2011; Chan *et al.,* 2010). It is also involved in maintaining the functioning of innate immune response. Several studies have reported that mutations in EGFR can lead to various types of cancers (Quatrale *et al.,* 2011). Shyh *et al.,* in 2007 targeted this EGFR in lung cancer cells with specific siRNA and designed the assembly by utilizing the NP technology. He used a carrier DNA along with lipids and polyethylene glycol (so as to make the NP inert to the immune system) in the NP enclosing the siRNA. The innovative addition was that of protamine and anisamide ligand. Protamine enhances the efficiency of NP by helping the separation of enclosed siRNA from NPs at the targeted site of delivery. This is important as studies have reported that siRNA assemblies will not work unless the siRNA is separated efficiently from the assembly components at site of action (D. Reischl *et al.,* 2010). The anisamide ligand helps to enhance the siRNA uptake by the cells thereby increasing the efficiency of the RNA interference pathway. The effect of these NPs was studied in the NCI-H460 xenograft tumor (lung tumor) bearing mice. Tumor shrinkage was reported to be due to the enhanced tumor cell apoptosis.

Aptamers are a promising new class of targeting agents (Mayer *et al.,* 2011; Levy *et al.,* 2008; Ozpolat *et al.,* 2010; Li *et al.,* 2011). Aptamers are globular molecules formed of modified oligonucleotides. The high affinity, specificity and presence of resistance conferring modifications against serum and tissue nucleases makes aptamers a promising tool (Hicke *et al.,* 2000). An exciting study was conducted by James et al demonstrating the efficient use of aptamer- siRNA chimeric RNAs for the treatment of prostate cancers in xenograft model of prostate cancer (McNamara *et al.,* 2006). Aptamers were employed as a delivery system of siRNA targeting the genes involved in providing the necessary survival signal to the tumor cell for the tumor progression. The siRNA targeting the genes i.e. polo-like kinase 1 (PLK1) and BCL2 gene were coupled to the aptamers targeting a protein specifically expressed over the prostate cancer cells and tumor vascular endothelium i.e. Prostate-specific membrane antigen (PMSA). These aptamers-coupled siRNA were termed as the aptamer-siRNA chimeras. The aptamer-siRNA chimeras were evaluated in mouse xenograft prostrate cancer model and it was found that their binding was target specific and the targeted genes i.e. polo-like kinase 1 (PLK1) and BCL2 were significantly silenced hence providing a new gateway for the treatment of the prostate cancer.

Polyelectrolyte micelle complex based siRNA delivery system is another exciting proceeding towards the therapeutic utilization of siRNA in cancer (Parveen *et al.,* 2011). Sun et al reported the use of such siRNA delivery system for chemotherapeutic purposes. They utilized vascular endothelial growth factor (VEGF) siRNA and conjugated it with Polyethylene glycol. The PEG siRNA was further exposed to polyethlenimine (PEI) which resulted in spontaneous formation of nanoscale polyelectrolyte complex micelles. The final structure of the nanoparticle included an inner core of siRNA-Polyethylenimine and Polyelectrolyte micelle complex and this inner core was surrounded by polyethylene glycol sheet. Since angiogenesis has been implicated in metastasis and vascular endothelial growth factor (VEGF) has a direct role in cancer prognosis. Many VEGF blockers have been designed so far. In line with this, the in *vivo* VEGF suppression through the use of siRNA- based nanotechnology has been reported to cause reduced tumor size demonstrating the promising use of siRNA based therapy for treating cancer (Kim *et al.,* 2008).

Another utilization of siRNA is in the treatment of numerous inflammatory disorders such as those induced by tumor necrosis factor alpha (TNF-α). The major contribution in the pathogenesis of such disorder is played by macrophages and microglia cells (Glass *et al.,* 2010; Geissmann *et al.,* 2010). Sang et al in 2010 attempted to treat such a condition. In this study, he utilized the siRNA approach to cause suppression of TNF-α in macrophage and microglia cells and then evaluated its effectiveness in pathological state progression. It was found that such an intervention significantly reduced lipopolysacharide (LPS) induced neuro-inflammation and neuronal apoptosis in *vivo* system. The composition of the strategy included short nicotinic acetylcholine receptor (AchR) binding peptide derived from the rabies virus glycoprotein (RVG) which was used as targeting legend because the macrophage and microglia cells express (AchR) receptor on their surface. This peptide was further used for fusion with the nona-D-arginine residues (RVG-9dR) to enable siRNA- binding. Later on it was found that RVG-9dR did the target specific delivery of siRNA to induce gene silencing in macrophages and microglia cells from wild type, but not AchR-deficient mice indicating the effectiveness of RVG-9Dr being a way to deliver the siRNA to macrophage and microglia and hence suppressing the TNF-α and

suppressing the neuroinflammatory disorder`s pathogenesis (Kim *et al.,* 2010).

Dendritic cells (DC) are known for their functional dichotomy and are thought to be a cell population which joins together the two arms of immunity i.e. the innate and adaptive immunity (Iwasaki *et al.,* 2010; Schenten *et al.,* 2011; Van *et al.,* 2007). DC has the potential to stimulate and discriminate the naive T cells into any one of these population i. e T helper (Th) 1, Th2 cells and the T-regulatory (T reg) cells (Steinman *et al.,* 2006; Maldonado *et al.,* 2001; Belz *et al.,* 2002; Mahnke *et al.,* 2002; Min *et al.,* 2003) . However, the type of T-cell population generated by DC is determined by the expression of co-stimulatory molecules as well as certain cytokines (Kubach *et al.,* 2005). Th2 cell is dependent on the stimulation of naive T cell by IL-10 (Tuettenberg *et al.,* 2009; Ronet *et al.,* 2010) whereas Th1 production occurs in response to IL-12 produced by DC (O'Garra *et al.,* 2009). IL-10 is also implicated in the production of T-reg population (Heo *et al.,* 2009). This capability of DC to produce different subsets of T cells has largely been explored for its therapeutic utilization. Studies have been conducted with an aim to generate DCs with a specific phenotype that may interfere with the disease progression. Jonathan et al utilized siRNA strategy to target IL-12 *p35* and showed that IL-12 p70 production in bone marrow derived DC is significantly suppressed upon exposure to LPS and tumor necrosis factor alpha (TNF-α) with simultaneous increase in IL-10 production. These DC were then cultured with allogenic T cells, enhanced Th2 response was observed. This also led to the suppression of allostimulatory function of DC. It was further supported by another study reporting that siRNA treatment has resulted in the production of Th2 response promoting DCs by exposing them to keyhole limpet hemocyanin (KLH). This proves that siRNA can be utilized to produce the desired type of DC which can further be manipulated for intervening the disease processes (Hill *et al.,* 2003).

After reviewing some important advancement in the field of RNAi based therapies, we can say that RNAi is a promising new therapeutic modality. siRNA designed against various culprit genes have proven to be a good therapeutic intervention in various in *vitro* and in *vivo* experiments. However, the safe and effective method to deliver siRNA remains challenging. As discussed already that in order to design a stable and targeted delivery system, various groups have employed various delivery system. Such RNAi-based immuno-therapies are mostly in clinical trials. The success rate of such clinical studies will determine the future of many and can bring a revolution in the treatment of diseases.

REFERENCES

1. Aigner, A. 2006. Gene silencing through RNA interference (RNAi) in vivo: strategies based on the direct application of siRNAs. J Biotechnol, 124: 12-25.
2. Belz GT, Heath WR and Carbone FR. 2002. The role of dendritic cell subsets in selection between tolerance and immunity. Immunol Cell Biol, 5:463-8.
3. Chan HL, Chou HC, Duran M, Gruenewald J, Waterfield MD, Ridley A and Timms JF. 2010. Major role of epidermal growth factor receptor and Src kinases in promoting oxidative stress-dependent loss of adhesion and apoptosis in epithelial cells. J Biol Chem, 285: 4307-18. Epub 2009 Dec 7.
4. Davis, ME. 2009. The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. Mol Pharm, 6: 659-68.
5. Dykxhoorn DM, Palliser D and Lieberman J. 2006. The silent treatment: siRNAs as small molecule drugs. Gene Ther, 13: 541-52.
6. Eckstein A, Grössl T, Geisler A, Wang X, Pinkert S, Pozzuto T, Schwer C, Kurreck J, Weger S, Vetter R, Poller W and Fechner H. 2010. Inhibition of adenovirus infections by siRNA-mediated silencing of early and late adenoviral gene functions. Antiviral Res, 88: 86-94.
7. Ganesan AK, Ho H, Bodemann B, Petersen S, Aruri J, Koshy S, Richardson Z, Le LQ, Krasieva T, Roth MG, Farmer P and White MA. 2008. Genome-wide siRNA-based functional genomics of pigmentation identifies novel genes and pathways that impact melanogenesis in human cells. PLoS Genet, 4(12): p e1000298.
8. Garrido G, Rabasa A, Sánchez B, López MV, Blanco R, López A, Hernández DR, Pérez R and Fernández LE.. 2011. Induction of immunogenic apoptosis by blockade of epidermal growth factor receptor activation with a specific antibody. J Immunol, 187: 4954-66. Epub 2011 Oct 7.
9. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M and Ley K. 2010. Development of monocytes, macrophages, and dendritic cells. Science, 327: 656-61.
10. Glass CK, Saijo K, Winner B, Marchetto MC and Fred H. 2010. Mechanisms underlying inflammation in neurodegeneration. Cell, 140: 918-34.
11. Heidel, JD. 2006. Linear cyclodextrin-containing polymers and their use as delivery agents. Expert Opin Drug Deliv, 3: 641-6.
12. Heo YJ, Joo YB, Oh HJ, Park MK, Heo YM, Cho ML, Kwok SK, Ju JH, Park KS, Cho SG, Park SH and Kim HY, Min JK.. 2009. IL-10 suppresses Th17 cells and promotes regulatory T cells in the CD4+ T cell population of rheumatoid arthritis patients. Immunol Lett, 127:150-6. Epub 2009 Nov 4.
13. Hicke BJ and Stephens AW. 2000. Escort aptamers: a delivery service for diagnosis and therapy. J Clin Invest, 106: 923-8.
14. Hill JA, Ichim TE, Kusznieruk KP, Li M, Huang X, Yan X, Zhong R, Cairns E, Bell DA and Min WP. 2003. Immune modulation by silencing IL-12 production in dendritic cells using small interfering RNA. J Immunol, 171: 691-6.
15. Hokaiwado N, Takeshita F, Banas A and Ochiya T. 2008. RNAi-based drug discovery and its application to therapeutics. IDrugs, 11: 274-8.
16. Iwasaki A and Medzhitov R. 2010. Regulation of adaptive immunity by the innate immune system. Science, 327: 291-5.

17. Kim SS, Ye C, Kumar P, Chiu I, Subramanya S, Wu H, Shankar P and Manjunath N. 2010. Targeted delivery of siRNA to macrophages for anti-inflammatory treatment. Mol Ther, 18: 993-1001. Epub 2010 Mar 9.
18. Kim SH, Jeong JH, Lee SH, Kim SW and Park TG. 2008. Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. J Control Release, 129: 107-16.
19. Koff JL, Shao MX, Ueki IF and Nadel JA.. 2008. Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium. Am J Physiol Lung Cell Mol Physiol, 294: 1068-75. Epub 2008 Mar 28.
20. Kumar, P, et al. 2008. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. Cell, 134: 577-86.
21. Kumar P, Ban HS, Kim SS, Wu H, Pearson T, Greiner DL, Laouar A, Yao J, Haridas V, Habiro K, Yang YG, Jeong JH, Lee KY, Kim YH, Kim SW, Peipp M, Fey GH, Manjunath N, Shultz LD, Lee SK and Shankar P. 2006. The RNA interference pathway: a new target for autoimmunity. Arthritis Res Ther, 8: 110.
22. Kubach J, Becker C, Schmitt E, Steinbrink K, Huter E, Tuettenberg A and Jonuleit H.. 2005. Dendritic cells: sentinels of immunity and tolerance. Int J Hematol, 81:197-203.
23. Lares MR, Rossi JJ and Ouellet DL. 2010. RNAi and small interfering RNAs in human disease therapeutic applications. Trends Biotechnol, 28: 570-9.
24. Li J, Tan S, Chen X, Zhang CY and Zhang Y. 2011. Peptide aptamers with biological and therapeutic applications. Curr Med Chem. 18: 4215-22.
25. Mahnke K, Schmitt E, Bonifaz L, Enk AH and Jonuleit H. 2002. Immature, but not inactive: the tolerogenic function of immature dendritic cells. Immunol Cell Biol, 80:477-83.
26. Maldonado-López R and Moser M.2001. Dendritic cell subsets and the regulation of Th1/Th2 responses. Semin Immunol, 5: 275-82.
27. Mayer G, Rohrbach F, Pötzsch B and Müller J. 2011. Aptamer-based modulation of blood coagulation. Hamostaseologie, 31: 258-63.
28. McNamara JO 2nd, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA and Giangrande PH. 2006. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras.Nat Biotechnol, 24: 1005-15.
29. Min WP, Zhou D, Ichim TE, Strejan GH, Xia X, Yang J, Huang X, Garcia B, White D, Dutartre P, Jevnikar AM and Zhong R. 2003. Inhibitory feedback loop between tolerogenic dendritic cells and regulatory T cells in transplant tolerance. J Immunol, 170: 1304-12.
30. Muñoz M, García-Erce JA and Remacha AF. 2011. Disorders of iron metabolism Part 1: molecular basis of iron homoeostasis. J Clin Pathol, 64: 281-6 Epub 2010 Dec 20.
31. O'Garra A and Murphy KM.2009. From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce TH1 development. Nature Immunology, 10; 929 –932.
32. Ozpolat B, Sood AK and Lopez-Berestein G. 2010. Nanomedicine based approaches for the delivery of siRNA in cancer. J Intern Med, 267: 44-53.
33. Parveen S, Misra R and Sahoo SK. 2011. Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. Nanomedicine, 2011 [Epub ahead of print].
34. Poliseno L, Mercatanti A, Citti L and Rainaldi G. 2004. RNA-based drugs: from RNA interference to short interfering RNAs. Curr Pharm Biotechnol, 5: 361-8.
35. Quatrale AE, Petriella D, Porcelli L, Tommasi S, Silvestris N, Colucci G, Angelo A and Azzariti A. 2011. Nti-EGFR monoclonal antibody in cancer treatment: in vitro and in vivo evidence. Front Biosci, 16: 1973-85.
36. Reischl D, Krenn JE, Bernhart E, Sattler W, and Zimmer A. 2010. Protamine Based siRNA-Nano particles: Particle Composition Dependant Influences on RNA Interference Efficiency in PNM16. Sci Pharm, 686.
37. Ronet C, Hauyon-La Torre Y, Revaz-Breton M, Mastelic B, Tacchini-Cottier F, Louis J and Launois P. 2010. Regulatory B cells shape the development of Th2 immune responses in BALB/c mice infected with Leishmania major through IL-10 production. J Immunol, 184: 886-94. Epub 2009 Dec 4.
38. Shimaoka, M. 2009. SiRNA-based therapeutics. Nippon Rinsho, 67: 1185-90.
39. Schenten D and Medzhitov R. 2011. The control of adaptive immune responses by the innate immune system. Adv Immunol, 109: 87-124.
40. Steinman RM and Hemmi H. 2006. Dendritic cells: translating innate to adaptive immunity. Curr Top Microbiol Immunol, 311:17-58.
41. Scherr M, Morgan MA and Eder M. 2003. Gene silencing mediated by small interfering RNAs in mammalian cells, Curr Med Chem. 10: 245-56.
42. Tang Y, Ge YZ and Yin JQ. 2007. Exploring in vitro roles of siRNA in cardiovascular disease. Acta Pharmacol Sin, 28: 1-9.
43. Tuettenberg A, Fondel S, Steinbrink K, Enk AH and Jonuleit H.2009. CD40 signaling induces IL-10-producing, tolerogenic dendritic cells. Experimental Dermatology, 19:44-53.
44. Van Vliet SJ, den Dunnen J, Gringhuis SI, Geijtenbeek TB and van Kooyk Y. 2007. Innate signaling and regulation of Dendritic cell immunity. Curr Opin Immunol, 19(4):435-40 Epub 2007 Jul 12.
45. Zheng X, Vladau C, Zhang X, Suzuki M, Ichim TE, Zhang ZX, Li M, Carrier E, Garcia B, Jevnikar AM and Min WP . 2009. A novel in vivo siRNA delivery system specifically targeting dendritic cells and silencing CD40 genes for immunomodulation. Blood, 113: 2646-54.

Original Article

Curcumin: It's Pharmacological and Therapeutic Properties
Running Head: Drug Delivery

Asif Nawaz*, Shefaat Ullah Shah, Kifayat Ullah Shah, Hashaam Akhtar**
Asim-Ur-Rehman, Abid Hussain, Gul Majid Khan
*Department of Pharmaceutics Faculty of Pharmacy Gomal University, Dera Ismail Khan (K.P.K) Pakistan
**Atta-ur-Rahman School of Applied Biosciences
drgulmajeed@yahoo.com

Abstract

Curcumin is a small molecular weight, polyphenolic compound, isolated from the roots of *curcuma longa* L. (family zingiberaceae), has been used traditionally for centuries in Asia for medicinal, culinary and other purposes. A large number of in vitro and in vivo studies in both animals and man have indicated that Curcumin has strong antioxidant, anti-inflammatory, anti-carcinogenic, anti-microbial, and anti-parasitic and other activities. The mechanisms of some of these actions have recently been intensively investigated. The compound inhibits the activity of growth factor receptors. The anti-inflammatory properties of curcumin are mediated through their effects on cytokines, lipid mediators, eicosanoids and proteolytic enzymes. Curcumin scavenges the superoxide radical, hydrogen peroxide and nitric oxide, and inhibits lipid peroxidation. These actions may be the basis for many of its pharmacological and therapeutic properties.

Keywords: Curcumin, *Curcuma longa*, anti-oxidant, anti-inflammatory, anti-cancer.

Introduction

Curcumin is a phenolic pigment of yellow color, obtained from crushed rhizome of C. longa Linn. Fig.1 (Family-Zingiberaceae) (Schmidt *et al*., 2007). It is the main constituent of oleoresin of turmeric. In crude extracts of rhizome of C. Longa, almost 70-76% of curcumin is present along with approximately 16 and 8 percent of demethoxycurcumin and bisdemethoxycurcumin respectively. Turmeric powder is used as a medicine to treat variety of diseases and extensively used for imparting flavor and color to eatables. Research on curcumin confirms a wide spectrum of therapeutic effects including antibacterial, antiviral, antispasmodic, antitumor, anti-inflammatory and hepatoprotective. Its efficacy in auto immune deficiency syndrome (AIDS) has been revealed in the last decade by different group of scientists (Araujo CAC and Leon LL., 2001; Maheshwari *et al*., 2006: Chattopadhyay *et al*., 2004).

The aim of this article is to invite the researchers to investigate the new curcumionid derivatives with chemical modifications, based in structure and biological activity relationship, in order to find new drugs that are less toxic to humans and also used for treatment of many diseases.

Chemical Properties of Curcumin

In Turmeric, Curcumin (1, 7-bis (hydroxyl-3-methoxyphenyl)-1,6- heptadiene-3, 5-dione) (Fig. 2), is a vital active ingredient responsible for its biological activity. It was first isolated in 1815, but it took another hundred years to elucidate its structure, which was solved in 1913. Curcumin is soluble in ethanol and acetone whereas insoluble in water. The naturally occurring ratios of curcuminoids in curcumin are about 80% curcumin, 15% demethoxycurcumin, and minute quantities of bisdemethoxycurcumin (Ireson *et al*., 2001).

Curcumin is comparatively unstable in phosphate buffer at pH 7.4 but the stability increases by either lowering the pH, or by augmenting it by glutathione, rat liver microsomes, *N* acetyl cysteine or ascorbic acid. Chemical synthesis of curcumin analogues results in various compounds with stronger anti-oxidant and tumor chemoprotective activities (Youssef *et al*., 2004).

Fig. 1. Source of curcumin (*C. longa*)

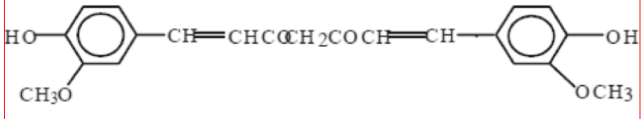

Fig. 2. Chemical structure of Curcumin

Curcumin Bioavailability and Pharmacokinetics

Various studies highlight the biotransformation of curcumin, which occurs in liver, kidney and GIT. It is first biotransformed to tetrahydrocurcumin and dihydrocurcumin and then these compounds are converted

Corresponding Author: Gul Majid Khan

to monoglucuronide (Lin *et al*., 2000). Thus the major metabolites of curcumin are tetrahydrocurcumin, dihydrocurcumin-glucuronide, tetrahydrocurcumin-glucuronide and curcumin-glucuronide.

The systemic bioavailability of Curcumin is not very high, so the pharmacological actions and activity of Curcumin may be displayed, in parts, by its metabolites. Hexahydrocurcuminol and hexahydrocurcumin are the major metabolites of Curcumin involved in the suspension of human hepatocytes, while Curcumin glucuronide and Curcumin sulfate are the predominant metabolites of Curcumin in human plasma *in vivo* (Ireson *et al*., 2002).

The reason behind the poor bioavailability of Curcumin is due to its rapid metabolism in the liver and intestinal wall. Piperine can increase the bioavailability of curcumin as it is a well-known inhibitor of hepatic and intestinal glucuronidation and in addition to this; it also increases the serum concentration, degree of absorption and thus the bioavailability of curcumin in homo sapiens (Shoba *et al*., 1998).

Mechanisms of Action

Antioxidant Effects

Water soluble and fat soluble extracts of turmeric and curcumin shows strong anti-oxidant activity as compared to vitamin C and E (Toda *et al*., 1985). Curcumin pretreatment decreases ischemia induced changes in the feline heart. An in vitro measuring the effects of curcumin on endothelial heme oxygenase-1 (inducible stress protein), was conducted utilizing bovine aortic endothelial cells. Enhanced cellular resistance to oxidative damage resulted with curcumin incubation (18 hrs) (Dikshit *et al*., 1995).

Hepatoprotective Effects

Turmeric has hepatoprotective properties similar to silymarin. Animal studies demonstrated turmeric's hepatoprotective effects from a variety of hepatotoxic insults, including carbon tetrachloride (CCl4), (Deshpande *et al*., 1998) galactosamine, acetaminophen (paracetamol), and Aspergillus aflatoxin. Hepatoprotective effect is mainly due to anti-oxidant property of turmeric. In rats acute and subactue liver injury was induced with CCl4, curcumin administration significantly decreased liver injury in rats as compared to controls. Turmeric and curcumin also reversed biliary hyperplasia, fatty changes and necrosis induced by aflatoxin production (Aspergillus parasiticus). Sodium curcuminate also exerts choleretic effects by increasing biliary excretion of bile salts, cholesterol and bilirubin, as well as increasing bile solubility. (Ramprasad C and Sirsi M., 1957).

Anti-inflammatory Effects

Curcumin of *curcuma long* shows potent anti-inflammatory effects (Chandra and Gupta S., 1972). Orally administered curcumin in instance of acute inflammation was found to be as effective as cortisone or phenylbutazone, and one half as effective in case of chronic inflammation. Oral administration of curcumin significantly reduced inflammatory swelling compared to control in rats with Freunds adjuvant-induced arthritis. In monkeys, curcumin inhibited neutrophil aggregation associated with inflammation (Srivastava R, 1989). *C. longa's* anti-inflammatory properties may be attributed to its ability to inhibit both biosynthesis of inflammatory prostaglandins from arachidonic acid and neutrophil function during inflammatory states. Curcumin may also be applied topically to the skin to counteract the inflammation and irritation associated with inflammatory skin conditions and allergies (Mukhopadhyay *et al*., 1982).

Anticarcinogenic Effects

Various studies including animals, invitro and humans show curcumin ability to inhibit carcinogenesis at three stages: tumor promotion, angiogenesis and tumor growth (Thaloor *et al*., 1998: Limtrakul *et al*., 1997). Curcumin inhibited tumor growth and cell proliferation, shown in studies of colon and prostate cancer. Curcumin is also capable of suppressing the activity of several common mutagens and carcinogens in a variety of cell types in both invitro and invivo studies (Mehta R.G. and Moon R.C., 1991). The anticarcinogenic effects of turmeric and curcumin are due to direct antioxidant and free-radical scavenging effects, as well as their ability to indirectly increase glutathione levels, thereby aiding in hepatic detoxification of mutagens and carcinogens, and inhibiting nitrosamine formation (Pizorrno and Murray M.T., 1999).

Antimicrobial Effects

Turmeric extracts and essential oils of curcuma longa inhibit the growth of variety of bacteria, fungi, and parasites. A study of chicks infected with parasite *Eimera maxima* demonstrated that diets supplemented with 1% turmeric resulted in a reduction in small intestinal lesions scores and improved weight gain (Allen *et al*., 1998). Another animal study, in which guinea pigs were infected with either dermatophytes, pathogenic molds, or yeast, found that topically applied turmeric oil inhibited dermatophytes and pathogenic fungi, but neither curcumin nor turmeric oil affected the yeast isolates. Improvements in lesions were observed in the dermatophyte- and fungi-infected guinea pigs, and at seven days post-turmeric application the lesions disappeared. Curcumin has also been found to have moderate activity against *Plasmodium falciparum* and *Leishmania major* organisms (Rasmussen *et al*., 2000).

Cardiovascular Effects

Lowering the triglyceride and cholesterol levels, inhibiting platelet aggregation and reducing propensity of low density lipoprotein (LDL) to lipid peroxidation are some of the turmeric's protective effects on the cardiovascular system (Srivastava R *et al*., 1984). Even with low doses of turmeric produces these effects. A study of eighteen atherosclerotic rabbits given low-dose (1.6-3.2 mg/kg body weight daily) turmeric extract long-establish decreased susceptibility of LDL to lipid peroxidation, as well as lowering the triglyceride and plasma cholesterol levels. Higher doses did not decrease lipid peroxidation of LDL,

on the other hand decrease in the levels of cholesterol and triglyceride were noted, although to a lesser extent than with the lower dose (Ramirez-Tortosa *et al*., 1999).

The mechanism of action of the effect of turmeric extract's on cholesterol levels is doubted to be due to reduced cholesterol uptake in the intestines and better conversion of cholesterol to bile acids in the liver. Potentiation of prostacyclin synthesis and inhibition of thromboxane synthesis are thought to be the reason behind the inhibition of platelet aggregation by *C. longa* constituents (Srivastava *et al*., 1984).

Clinical Indications

Hepatoprotection, Cholelithiasis, and Cholestasis

Hepatoprotective effects of turmeric are seen in a number of animal studies, suggesting that it is a strong candidate to be used in cases of toxic insult caused by exogenous toxins from lifestyle and living environment. Curcumin may also be helpful in treating gallstones, as it has also shown choleretic activity in different studies. (Deshpande *et al*., 1998).

Inflammation

Curcumin is a strong anti-inflammatory agent with specific COX-2 inhibiting and lipoxygenas properties. It effectively reduces both acute and chronic inflammations *in vitro* and *in vivo* studies (Srivastava R., 1989). In a crossover, placebo-controlled study of forty-two patients with osteoarthritis, a combination product containing turmeric, zinc, *Boswellia serrate* and *Withania somnifera* were used and after three months on the combination or placebo, patients noted a significant relief in pain ($p<0.001$) and disability ($p<0.05$) (Kulkarni *et al*., 1991).

Cancer

Various studies demonstrated that turmeric shows some anticarcinogenic effects. Whereas its flavonoids component curcumin shows its effects against colon, prostate and breast cancers, in addition to this, it also works against melanomas (Chauhan DP, 2002: Reddy BS and Rao CV, 2002: Ramachandran C. *et al*., 2002: Somasundaram S. *et al*., 2002). A study conducted on twenty-five patients with high risk of premalignant lesions or neoplasia showed histologic improvement in two of seven patients with oral leukoplakia, one of two patients with resected bladder cancer, one of four patients with cervical intraepithelial neoplasm, one of six patients with intestinal metaplasia of the stomach and two of six patients with Bowen's disease. Further studies need to be performed to elucidate the potential of this natural product in treating or preventing cancers (Somasundaram S. *et al*., 2002).

Hyperlipidemia

Numerous studies show that turmeric is effective in decreasing the lipid concentration in blood but less is known to be able to confirm these findings and utilize this therapeutic activity in our daily lives, so further clinical studies and experiments need to be designed in this area to discover optimal dosage and efficacy for cardiovascular protection and lipid lowering (Ramirez-Tortosa *et al*., 1999).

Gastric Ulcer

A study on patients with endoscopically diagnosed peptic ulcer showed promising results of turmeric as an antiulcer drug. Participants were given 600mg of powdered turmeric five times a day and ulcer was completely healed in 48% of patients after a 4 week time. The efficacy of turmeric increased over time and no significant adverse reaction or blood abnormalities were seen (Prucksunand *et al*., 2001).

Chronic Anterior Uveitis

Curcumin is also found to be effective in corticosteroid therapy, results of a study conducted on 32 patients with chronic anterior uveitis, who were given 375mg of Curcumin thrice a day for 12 weeks showed promising results and Curcumin was found effective in 86% of individuals (Lal B. *et al*., 1999).

Conclusions

Curcumin is a natural substance with many pharmacological and therapeutic activities, some of which have been experimentally and clinically utilized in both man and animals. Notable among these are the antioxidant, anti-inflammatory and anti-carcinogenic properties, all three of which seem to be interrelated. It is encouraging that Curcumin is of low toxicity. Despite a plethora of phytochemical, pharmacological, biochemical and toxicological data on Curcumin, large well-designed clinical trials and epidemiological data are warranted to substantiate it usefulness in the treatment and/or prevention of cancer, rheumatoid arthritis and other conditions of human patients.

REFERENCES

1. Allen PC, Danforth HD, Augustine PC. 1998. Dietary modulation of avian coccidiosis. Int J Parasitol. 28:1131-1140.
2. Araujo C A C and Leon L L. 2001. Biological activities of Curcuma longa L. Mem. Inst. Oswaldo Cruz. Riode Janeiro, 96: 723 - 728.
3. Chandra D and Gupta S. 1972. Anti-inflammatory and anti-arthritic activity of volatile oil of Curcuma longa (Haldi). Indian J Med Res. 60:138-142.
4. Chattopadhyay I, Biswas K, Bandypadhyay U and Banerjee R K. 2004. Turmeric and curcumin: biological actions and medicinal applications. Cur. Sci. 87: 44 - 53.
5. Chauhan DP. 2002. Chemotherapeutic potential of curcumin for colorectal cancer. Curr Pharm Des. 8:1695-1706.
6. Deshpande UR, Gadre SG and Raste AS. 1998. Protective effect of turmeric (Curcuma longa L.) extract on carbon tetrachloride-induced liver damage in rats. Indian J Exp Biol. 36:573-577.
7. Dikshit M, Rastogi L, Shukla R and Srimal RC. 1995. Prevention of ischaemia-induced biochemical changes

by curcumin and quinidine in the cat heart. Indian J Med Res. 101:31-35.
8. Ireson C, Orr S and Jones DJL. 2001. Characterization of metabolites of the chemopreventative agent curcumin in rat and human hepatocytes and in rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. Cancer Research. 61: 1058-1064.
9. Ireson CR, Jones DJ, Orr S, Coughtrie MW, Boocock DJ, Williams ML, Farmer PB, Steward WP and Gescher AJ. 2002. Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. Cancer Epidemiol Biomarkers Prev. 11(1): 105–111.
10. Kiso Y, Suzuki Y and Watanabe N. 1983. Antihepatotoxic principles of Curcuma longa rhizomes. Planta Med. 49:185-187.
11. Kulkarni RR, Patki PS, Jog VP, Gandage SG and Patwardhan B. 1991. Treatment of osteoarthritis with a herbomineral formulation: a double-blind, placebo-controlled, cross-over study. J Ethnopharmacol. 33:91-95.
12. Lal B, Kapoor AK, Asthana OP, Agrawal PK, Prasad R, Kumar P and Srimal RC. 1999. Efficacy of curcumin in the management of chronic anterior uveitis. Phytother Res. 13:318-322.
13. Limtrakul P, Lipigorngoson S and Namwong O. 1997. Inhibitory effect of dietary curcumin on skin carcinogenesis in mice. Cancer Lett. 116:197-203.
14. Lin JK, Pan MH and Lin-Shiau SY. 2000. Recent studies on the biofunctions and biotransformations of curcumin. Biofactors. 13(1–4): 153–158.
15. Maheshwari R K, Singh A K, Gaddipati J and Smiral R C. 2006. Multiple biological activities of curcumin: a short review. Life Sci. 78: 2081 - 2087.
16. Mehta RG and Moon RC.1991. Characterization of effective chemopreventive agents in mammary gland in vitro using an initiation-promotion protocol. Anticancer Res. 11:593-596.
17. Mukhopadhyay A, Basu N and Ghatak N. 1982. Anti-inflammatory and irritant activities of curcumin analogues in rats. Agents Actions. 12:508-515.
18. Park EJ, Jeon CH, Ko G, Kim J, Sohn DH. 2000. Protective effect of curcumin in rat liver injury induced by carbon tetrachloride. J Pharm Pharmacol. 52:437-440.
19. Pizorrno JE and Murray MT. 1999. Textbook of Natural Medicine, 2nd Ed. London: Churchill Livingstone. 689-693.
20. Prucksunand C, Indrasukhsri B, Leethochawalit M and Hungspreugs K. 2001. Phase II clinical trial on effect of the long turmeric (Curcuma longa Linn) on healing of peptic ulcer. Southeast Asian J Trop Med Public Health. 32:208-215.
21. Ramachandran C, Fonseca HB, Jhabvala P, Escalon EA, Melnick SJ. 2002. Curcumin inhibits telomerase activity through human telomerase reverse transcritpase in MCF-7 breast cancer cell line. Cancer Lett. 184:1-6.
22. Ramirez-Tortosa MC, Mesa MD and Aguilera MC. 1999. Oral administration of a turmeric extract inhibits LDL oxidation and has hypocholesterolemic effects in rabbits with experimental atherosclerosis. Atherosclerosis. 147:371-378.
23. Ramprasad C, Sirsi M. Curcuma longa and bile secretion. Quantitative changes in the bile constituents induced by sodium curcuminate. J Sci Indust Res 1957;16C:108-110.
24. Rasmussen HB, Christensen SB, Kvist LP and Karazami A. 2000. A simple and efficient separation of the curcumins, the antiprotozoal constituents of Curcuma longa. Planta Med. 66:396-398.
25. Reddy BS and Rao CV. 2002. Novel approaches for colon cancer prevention by cyclooxygenase-2 inhibitors. J Environ Pathol Toxicol Oncol. 21:155-164.
26. Schmidt BM, Ribnicky D M, Lipsky P E and Raskin I. 2007. Revisiting the ancient concept of botanical therapeutics. Nature Chemical Biology. 3: 360 – 366.
27. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R and Srinivas PS. 1998. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med. 64(4): 353–336.
28. Somasundaram S, Edmund NA and Moore DT. 2002. Dietary curcumin inhibits chemotherapy-induced apoptosis in models of human breast cancer. Cancer Res. 62:3868-3875.
29. Srivastava R, Puri V, Srimal RC and Dhawan BN. 1986. Effect of curcumin on platelet aggregation and vascular prostacyclin synthesis. Arzneimittelforschung. 36:715-717.
30. Srivastava R. 1989. Inhibition of neutrophil response by curcumin. Agents Actions. 28:298-303.
31. Thaloor D, Singh AK and Sidhu GS. 1998. Inhibition of angiogenic differentiation of human umbilical vein endothelial cells by curcumin. Cell Growth Differ. 9:305-312.
32. Toda S, Miyase T and Arich H. 1985. Natural antioxidants. Antioxidative compounds isolated from rhizome of Curcuma longa L. Chem Pharmacol Bull. 33:1725-1728.
33. Youssef KM, El-Sherbeny MA, El-Shafie FS, Farag HA, Al-Deeb OA and Awadalla SR. 2004. Synthesis of curcumin analogues as potential antioxidant, cancer chemopreventative agents. Archiv der Pharmazie (Weinheim). 337: 42-54.

Original Article

Knowledge Regarding Spread, Diagnosis and Treatment of HCV Patients among Primary Health Care Physicians in Islamabad and Rawalpindi

Najma Javed, Sumera Naz
Pakistan Medical Research Council PMRC Islamabad.
Javed.najma@gmail.com

Abstract

To explore the basic knowledge of primary health care doctors on transmission routes, risk factors and management of HCV infection. A cross-sectional facility-based study of six month (Dec.2008-June 2009) duration. Universal sampling technique. Thirty three Basic Health Units (BHUs) in Islamabad and Rawalpindi districts with their attached 07 dispensaries were surveyed. A total of 40 Primary Health Care Physicians (PHCP) from two cities (Islamabad and Rawalpindi) were interviewed. A pre tested questionnaire with multiple choices was used to record their knowledge on transmission routes, causative factors and management of this infection. A total of 40 primary care physicians were interviewed. There was poor knowledge about modes of disease transmission while diagnosis and treatment was well known in majority. The frequency of disease transmission to neonate and the time of checking the child in case of a HCV positive mother were not known by many GPs, which need to be taken seriously specially in our setting where GP is the first or second line person that is approached by the patient. Majority of the physicians knew that HCV is not transmitted through breast feeding. Genotyping is done by about 61% physicians thus adding a very expensive test with very limited use. About 60% GPs counsel the patients that are found positive for the test, which is a good sign. The study identified a strong need for continuing education program for the primary care physicians on HCV infection.

Introduction

Hepatic infection with C virus (HCV) is a major health problem worldwide. Most of the developed countries have prevalence of anti-HCV between 1% to 2% (Di Bisceglie 1998). In US chronic infections transmitted through blood, HCV is the commonest one (1998). This chronic infection is responsible for approximately 20% cases of acute hepatitis, 70% of chronic hepatitis and 30% of fatal liver disease (Crawford 1997). In a recent study, prevalence of anti HCV in Pakistan is 5% (2009). While another study in NWFP showed that prevalence of HBsAg was 3.5% and anti HCV was 13.8% giving the combined prevalence of 17.3% (Ahmad, 2009) on the whole.

Excess of published information on various aspects of hepatitis C virus is currently available. In Pakistan many studies have been done on awareness and practices about AIDS and viral hepatitis in various population/ groups and most studies have shown deficient awareness in all groups including health care professionals (1998). Awareness about hepatitis C is crucial because currently there is no vaccine available for its prevention and persons with chronic infection play crucial role in spread of disease to others and are at a risk for developing chronic viral illness (Di Bisceglie 1998). More over doctor to patient and vice versa transmission is also reported (Goujon 2000). Primary health care physicians, who are major part of health care delivery system, must have basic information on these infections as these doctors have key role in stopping disease spread and identifying those from vulnerable groups at the earliest before they develop fatal complications (2000).

The present study was done to explore the extent of information of health care personnels on transmission, diagnosis of hepatitis C infection and proper management of such patients.

Objective

The study was done to determine the extent of information of primary health care providers related to the transmission, diagnosis and treatment of patients.

Methods

Study design: A cross-sectional study with universal sampling technique.

Study area / setting: The study was conducted in 33 basic health Units of district Islamabad and Rawalpindi. A total of 13 BHUs (Basic Health Units) of Islamabad and 20 BHUs from Rawalpindi were surveyed. Some dispensaries of Federal Government Services Hospital Islamabad were also included in the study to achieve the target sample size.

Sample size: A total of 40 Primary Health Care Physicians were interviewed from 33 BHUs and 07 Government dispensaries in six months.

Inclusion Criteria: Only Primary health care physicians in public sector were enrolled in the study.

Exclusion Criteria: All private physicians were excluded along with PHCP who refused to give consent for the study.

Corresponding Author: Sumaira Naz
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Methods

Primary Health care Physicians (PHCP) from two cities (Islamabad and Rawalpindi) in Pakistan were enrolled in the study and interviewed. Information was recorded on pretested proforma which comprised of close ended questions with multiple choices. Demographic variables with total duration of overall practice period were recorded. Data was also collected on availability of diagnostic services at the BHUs along with physician's knowledge on the routes of transmission of HCV, its diagnostic tests, genotypes, information regarding HCV in pregnancy and lactation, its treatment and side effects and finally counseling regarding the disease.

Usually at each BHU one medical doctor is deputed while at Rural Health Centers (RHCs) 2-3 doctors were working. After taking their informed consent, the questionnaire was distributed to PHCPs at their facilities. Interviewer briefed the facility incharges on importance of study for 10 minutes. To respond to any query raised by respondent, availability of the researcher was ensured at the time of interview. One researcher was present during the survey administration to answer queries raised by respondents. Interview took about half an hour. Data was entered on computer Excel spread sheet for analysis.

Results

There were 22 female and 18 male doctors who were interviewed. The ages of interviewees were recorded to be 27 to 58 years. Their median age was 40.2 years. The average number of years spent in the practice was 12.8 years. Diagnostic facilities for HCV rapid testing kits (Agglutination method) were available in 15 (37.5%) health care centers. To refresh their knowledge 25% respondents review related books once in a week time while only 20% used internet to get latest information.

Only 27.5% of the PHCPs knew that HCV spreads through blood and body secretions and 5% knew that the virus could survive outside the human body for over 12 hours at room temperature and yet can infect others. Only 35% physicians knew the actual dilution of chlorine that was used in their facility to disinfect the surfaces against HCV infection. All respondents knew that tattooing is also a source of HCV transmission.

Knowledge about the diagnosis of infection was adequate in most PHCPs. Though 70% physicians test their patients for anti-HCV within three months of exposure but only 30% knew that patients could be tested by PCR within 1-2 weeks of exposure. Majority (90%) of physicians had knowledge that even in presence of normal Liver Function Tests (LFTs) patients could have chronic HCV infection. A list was provided of the high risk individuals who should be tested for HCV, 95% physicians could identify the high risk individuals who should be screened. After diagnosis 85% of the PHCPs referred their patients to Hepatologist for the treatment.

Majority (87.5%) of respondents said that they test antenatal women for HCV, while others did not. Knowledge about the disease transmission through breast milk was poor in 35% physicians as they stopped the mother from breast feeding the child. Similarly 52% physicians knew that a high viremic mother could transmit the disease to her newborn. Almost all (95%) PHCPs did not know when to test the baby born to a HCV positive mother and 52% did not know when to treat the child.

Regarding genotyping, 85.5% of PHCP knew about genotypes of HCV and 77.5% thought it is necessary to know the genotype before starting treatment for HCV.

Out of 40 PHCPs, 85% knew that HCV positive patient should be vaccinated against HBV. Knowledge about dietary intake in HCV was poor in 65% of the physicians as they restricted their patients from eating fatty and protein rich diet and only 35% allowed them a normal diet.

After diagnosis 85% of the PHCPs referred their patients to Hepatologist for the treatment. Out of 40 respondents, 37.5% were not aware of the treatment for HCV while 62.5% knew right treatment is with interferon and ribavirin. Regarding treatment of HCV in children only 20% knew that interferon should not be given to the children of less than 5 years of age. Similarly there was knowledge gap in tests to be done during and after treatment of HCV.

Discussion

This study explored the level of knowledge of primary care physicians regarding HCV spread, diagnosis and treatment along with gaps. Similar results were reported from France and America where general doctors knowledge about hepatitis C was not upto the mark (Fattovich 2003, Ouzan *et al*. 2003a, Ouzan *et al*. 2003b).

Another study conducted in Turkey on approach of general physicians to diagnose and treat viral hepatitis indicated that GPs were well informed of various risk groups and the transmission routes of HBV and HCV infections but knowledge on when to test for viral hepatitis and which is the appropriate treatment for this infection, considerable gaps were found. These findings are consistent with our results. However in our study knowledge about survival of the virus outside the human body was quite deficient. The Turkish study further found that in primary health care centers they had insufficient knowledge about correct diagnoses and follow up of the patients (Shehab *et al*. 2001).

In the present study knowledge about disease transmission, diagnostics that can be used for early detection, maternal to child transmission were deficient in many physicians, as 70% physicians were not aware that PCR can detect infection within 1-2 weeks. On the other hand 52.5% responded knew about reduced vertical transmission of HCV as compare to HBV.

Only 15% health care centers had diagnostic facilities for HCV testing. Yildiz *et al* also reported that only a few health facilities had diagnostic for viral hepatitis (Peksen *et al*. 2004). These finding suggest that lack of availability of diagnostic facilities for this infection may cause wrong diagnosis, multiple tests and over ambitious treatment.

In our study population 72.5% physicians had poor information on spread of HCV where as only 27.5% could tell that transfusion of blood or blood products, organ transplantation, sexual intercourse, intravenous drug use, infected HCV mother and hemodialysis are possible routes

of transmission for HCV. A study conducted in Pakistan showed that 56% of surgeons had no knowledge about the fact that disease could be spread by unprotected sex, 82% did not know about vertical transmission. On the other hand 93% knew that infected blood could spread the illness and 88% knew that a needle-stick injury is a risk factor (Rana *et al*. 2000).

In present study respondents had good knowledge about HCV genotypes and 82.5% were aware of term genotype. Majority (77.5%) of the physicians were agreeing that HCV patients genotyping is necessary in management of infection while 22.5% did not. Information about genotypes is important for clinical progress and especially to monitor the response in interferon treatment (Raja and Janjua 2008). Over 80% cases have genotype 3 in Pakistan, therefore the use of routine genotyping is not recommended as it is a very expensive test. It should be checked in individuals who have a chance of getting infected from a non Pakistani strain of the virus. The present study therefore shows that a lot majority of physicians are wasting a large amount of patient's money on a very non significant test. Majority of the physicians (70%) knew that a person can be infected with more than one HCV genotype at a time, however 90% of them could not tell the reason that why do most of the patients remain infected despite of treatment.

Manns *et al* also reported that required information for HCV treatment were found poor, reason might be advancement in this field. In their study all of the practitioners were unaware of pegylated interferon as treat option (Fried *et al*. 2002, Manns *et al*. 2001) and only 3 were aware of importance of combination therapy. Five general practitioners believed interferon monotherapy as right treatment option where as other two suggested treatment with lamivudine and interferon. In contrast to the above report, in the present study, 62.5% physicians had knowledge that combination therapy with interferon and ribavirin is the treatment of choice for HCV infection where as 37.5% PHCPs could not tell the current treatment and 7.5% respondent told that Ribavirin alone is a treatment of choice. Same findings were reported by d'Souza *et al* where primary health care physicians had no knowledge on the current treatment for hepatitis c and also a significant number of these doctors could not infer anti HCV results (D'Souza *et al*. 2004). This is in contradiction to results found in our study where majority of our PHCPs were aware of diagnostic tests for HCV and about its chances of being false positive and false negative.

In a study conducted by Zanetti most GPs believed that vertical route is common mode of transmission for HCV therefore to avoid possible transmission, GPs asked mothers not to breast feed (Resti 1999). However all GPs wanted routine screening of all pregnant women for HCV. Thomas *et al* in their study found that though there is only 6% risk of transmission through vertical route, over 80% blamed this route to be most important causative factor for HCV transmission (Shehab *et al*. 1999). In our study 52.5% of the respondents knew that as compared to HBV infection, materno fetal transmission of HCV is less (5%) and 87.5% routinely tested pregnant patients for HCV infection, though no international or national guidelines recommend testing of pregnant cases for HCV. As far as breast feeding is concerned though majority (65%) advised their HCV positive mothers to continue breast feeding to their newborns but still 35% stopped it due to fear of disease transmission. The findings of the survey conducted in USA on risk factors, management and attitude on diagnosis for hepatitis C by health care providers indicated diagnosis and referral for hepatitis C patients may not be at optimal level (Shehab *et al*. 2001). Our Findings are different and 90% of PHCPs suspect HCV infection even though LFTs are normal and 85% send HCV positive patients to the specialist after diagnosis for further management. In the specialized era, it has been observed that specialists doctors are made responsible to treat patients with HCV infection. As patients don't know the extent of their illness so they usually go to these first level physiscians for diagnosis and treatment, so it is important that their level of knowledge should be advance enough to filter patients with HCV and could timely refer them to the specialist. Due to the large bulk of patients and low number of specialists patients have to wait longer to get services that aggravate patient's worries as well their family doctors. In this scenario it becomes essential to change the mind set of doctors at primary health care level for HCV infection, its diagnosis and treatment. Needless to say that primary health care physician are the first who interact with infected individuals, so they should have knowledge and basic training on diagnosis, the latest treatment for HCV infection. In addition they must know reppropriate time for referral (where and to whom) for such patients. To keep them upraised about common ailements and their treatment, they need to undergo regular training and medical education.

Conclusions

Our PHCPs recognize their shortcomings. During our survey all emphasized need for training on HCV management especially in children. Continued medical education and disease specific protocols are required for GPs are required to develop the capacity of this cadre for the best service deliver for HCV infected individuals for quality labs and referral mechanism at primary health care level.

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REFERENCES

1. Morbidity and Mortality Weekly Report. Recommendations for prevention and control of hepatitis C virus (HCV) and infection and HCV-related chronic disease 1998. Report no. MMWR; 47: No. RR-19.
2. 2000. Hepatitis C--global prevalence (update). Wkly Epidemiol Rec 75: 18-19.
3. 2009. Prevalence of Hepatitis B&C in Pakistan.
4. Ahmad A AB, Ali A, Ahmad Y. 2009. Seroprevalence of HBsAg and anti-HCV in general healthy population

of Swat district with frequency of different HCV Genotypes. Pakistan Journal of Medical Sciences 25: 744-748.

5. Christophe P. Goujon VMS, Jaouad Grofti, Joëlle Montigny, Vincent Jeantils, Pascal Astagneau, Willy Rozenbaum, Florence Lot, Claudie Frocrain-Herchkovitch, Nathalie Delphin, Frédéric Le Gal, Jean-Claude Nicolas, Michel C. Milinkovitch and Paul Dény 2000. Phylogenetic Analyses Indicate an Atypical Nurse-to-Patient Transmission of Human Immunodeficiency Virus Type 1. Journal of Virology 74: 2525-2532.
6. Crawford JM. 1997. The liver and the biliary tract. Pages 516-555 in Kumar V CR, Robbins SL, , ed. Basic Pathology, Philadelphia: WB Saunders.
7. D'Souza RF, Glynn MJ, Alstead E, Osonayo C, Foster GR. 2004. Knowledge of chronic hepatitis C among East London primary care physicians following the Department of Health's educational campaign. QJM 97: 331-336.
8. Di Bisceglie AM. 1998. Hepatitis C. Lancet 351: 351-355.
9. Fattovich G. 2003. Natural history and prognosis of hepatitis B. Semin Liver Dis 23: 47-58.
10. Fried MW, *et al*. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347: 975-982.
11. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358: 958-965.
12. Ouzan D, Hofliger P, Cavailler P, Mamino C, Tran A. 2003a. [Screening and hepatitis C management survey in general medicine in the Alpes-Maritimes and east Var area]. Gastroenterol Clin Biol 27: 90-93.
13. Ouzan D, Cavailler P, Hofliger P, Mamino C, Joly H, Tran A. 2003b. [Modalities of care in anti HCV positive patients identified in General Medicine in the Alpes-Maritimes district]. Gastroenterol Clin Biol 27: 376-380.
14. Peksen Y, Canbaz S, Leblebicioglu H, Sunbul M, Esen S, Sunter AT. 2004. Primary care physicians' approach to diagnosis and treatment of hepatitis B and hepatitis C patients. BMC Gastroenterol 4: 3.
15. Raja NS, Janjua KA. 2008. Epidemiology of hepatitis C virus infection in Pakistan. J Microbiol Immunol Infect 41: 4-8.
16. Rana JS, Khan AR, Haleem AA, Khan FN, Gul A, Sarwari AR. 2000. Hepatitis C: knowledge, attitudes and practices among orthopedic trainee surgeons in Pakistan. Ann Saudi Med 20: 477-479.
17. Resti M. 1999. Mother-to-infant transmission of hepatitis C virus. Ital J Gastroenterol Hepatol 31: 489-493.

    Shehab TM, Sonnad SS, Lok AS. 2001. Management of hepatitis C patients by primary care physicians in the USA: results of a national survey. J Viral Hepat 8: 377-383.
18. Shehab TM, Sonnad SS, Jeffries M, Gunaratnum N, Lok AS. 1999. Current practice patterns of primary care physicians in the management of patients with hepatitis C. Hepatology 30: 794-800.

Original Article

Retrospective study of lymphadenopathy by FNAC in National Institute of Health Islamabad- Pakistan

Sumera Naz *, Arshad Mumtaz**
*PMRC- Central Research Center-Islamabad
**National Institute of Health-Islamabad
naz.sumera@gmail.com

Abstract

The objective of the study was to review the pathology of lymph node disorders in adults with peripheral lymphadenopathy. A five year (1998- 2003) retrospective study of lymph node Fine Needle Aspiration Cytology performed at Histopathology department of National Institute of Health Islamabad. Females constitute the major group with 130 cases (64%) in our study. Fifty four percent of cases were below 20 years of age. Benign lesions were found in 91% of the patients, the majority of which were tuberculous lymphadenitis (52%) followed by nonspecific reactive hyperplasia (39%). Frequency of metastatic carcinoma was 9(4%), malignant lymphoma 2(1.0%) and insufficient aspirate in 10 (5%) cases. The commonest site of lymphadenopathy was cervical (71%) followed by sub mandibular (11%), inguinal 8% and axillary 7%. Reactive hyperplasia was more frequent in female (62%) than male patients (38%), similarly tuberculous lymphadenitis was comparatively more frequent in female (64%) than male group (36%) our in study. Tuberculous lymphadenitis was the most common pathological diagnosis in young females (15-25years). It is concluded that the pattern of disease is similar to that of other countries of the region.

Key words: Fine Needle Aspiration Cytology, lymphadenopathy, tuberculous lymphadenitis

Introduction

Lymphadenopathy refers to nodes that are abnormal in size, consistency or number. Chronic lymphadenopathy might be caused by lymphomas and other malignancies. Lymphadenopathy is one of the commonest clinical presentations` of patients, attending the outdoor department. Aetiology varies from an inflammatory process to a malignant condition (Pandit *et al*., 1987). The causes of lymphadenopathy are extensive and include bacterial, viral and fungal infections.

Chronic peripheral lymph node enlargement in adults signifies an underlying disease and has continued to pose a diagnostic dilemma to physicians. Several studies in the developing countries highlight the tuberculosis and other infections as major etiological agents (Mandong *et al.*1999) while malignancies are the predominant cause of lymph node enlargement in developed countries. Primary tumors of the lymphatic tissue are quite common for example in Iraq, it accounts for 8% of the total cancer cases (Iraqi Center, 1993). Moreover, lymph nodes are a common site of metastasis for different cancers. Thus, clinical recognition and urgent diagnosis of palpable lymphadenopathy by FNA is of great importance.

Keeping in view, the plethora of diseases that may cause lymphadenopathy, it is essential to define the pattern of disorders presenting primarily as lymph node enlargement in a particular environment. The pattern of the disease is different in children and adults with non specific reactive hyperplasia as major cause in children with a developing immune system (Afridi *et al*.2005).

This study aims at defining the causes of peripheral lymphadenopathy and the pattern of lymph node distribution in adult patients seen in Histopathology department of National Institute of Health Islamabad.

Patients and Methods

Two hundred and four cases with Lymphadenopathy at various sites were subjected to FNAC technique at National Institute of Health Islamabad from Jan 1998 to Dec 2003. Clinicodemographic data regarding age, sex anatomical site, size, consistency, duration, family history, history of the previous similar lesion, and other relevant lab investigations (if available) were obtained from the departmental history proforma of each patient. The slides of selected cases were retrieved from the archives of the department.

Selection criteria: - Adults with 15 years of age or above suffering from peripheral lymphadenopathy.
Rejection criteria: - Improper fixation, drying artifact, air-dried sample, improperly labeled specimen and metastaic lymph nodes associated with evidence of primaries elsewhere in the body were excluded from the study.

Results

A total of 204 FNAC of lymph nodes were done from adults (15 year old and above) constituting 78% of total number of FNAC lymph nodes done in department during five year period of study (1998 to 2003).

Figure I shows sex distribution in 204 cases. Out of these, one hundred and thirty (64%) were of female while seventy four cases (36%) were of male, giving female to male of 1.7:1.

The cases were divided into five groups. The brief cytological criteria adopted for classification were as follows

1. Reactive hyperplasia: Smears revealed polymorphous population of lymphoid cells with predominance of

Corresponding Author: Sumera Naz

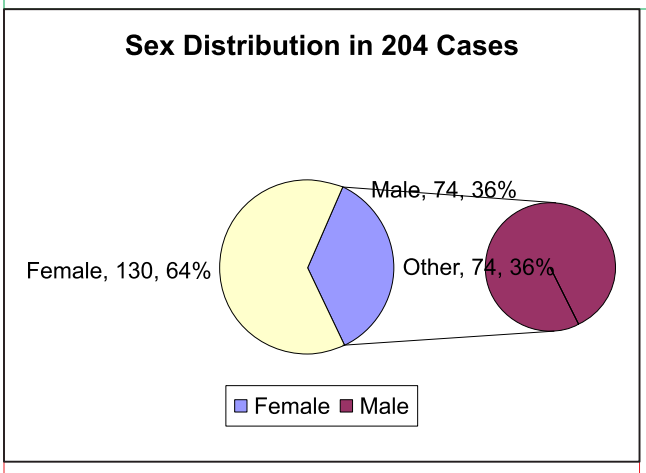

Fig I. Sex Distribution in 204 Cases

mature lymphocytes.

2. Consistent with Tuberculous Lymphadenitis: revealed caseous necrotic material, epitheloid cells, lymphocytes and an occasional giant cell in some cases.
3. Metastatic carcinoma: showed malignant cells, usually arranged in groups or clusters, along with other lymphoid cells.
4. Lymphoma: Non-Hodgkin's lymphoma showed a homogeneous pattern, consisting of atypical lymphoblasts or lymphocytes. Hodgkin's lymphoma showed a mix cell population with the characteristic Reed Sternberg giant cell.
5. Insufficient aspirate: smears revealed mainly hemorrhagic aspirate.

Figure II show the cytological results in 204 cases under study tuberculos lymphadenitis with one hundred and four cases (51%) was the most common pathologic type followed by reactive hyperplasia (39%), metastatic carcinoma (4%) and malignant lymphoma with one case (0.49%). FNA was non-diagnostic in 10 (5%) cases due to insufficient aspirate.

Figure III shows the distribution of cases under study as per their anatomical site. One hundred and forty four lymph nodes (70%) were of the cervical region in our study. Among the remaining cases, 22 (11%) were submandibular, 8 (3.9%) were axillary, 14 (6.9%) were clavicular and 16 (7.8%) were of inguinal region.

Table II shows the diagnosis according to the anatomical site of lymphadenopathy. The cases were divided into five groups. Out of these four were pathological groups while one i.e. insufficient aspirate was to evaluate the level of success of the procedure. Out of 141 cases of cervical region, reactive hyperplasia is in 57 cases while 80 suffered from chronic tuberculosis. Four patients were also suffering from the metastatic carcinoma in this group of our study. Out of 14 cases of axillary lymph node, nine suffered from the tuberculosis.

Table III shows the diagnosis according to the sex distribution in 260 cases under study. Seventy four cases are of male while 130 are of female patients. 18.1% males while 32.8% females were suffering from tuberculous lymphadenitis. Both in males and female group, reactive hyperplasia is the second commonly found pathology with 30 (14.7%) and 50 (32.8%) cases. The pattern of incidence is somewhat similar in female group but here the insufficient aspirate is 6.5% while it is 3.9% in male group. In female group 1.9% cases are also of malignant lymphoma.

Figure IV shows the cytological findings of the cases under study as per their age groups. Reactive hyperplasia was predominant cause of lymphadenopathy in age group 15-25 years while tuberculosis was dominant in age group 25-35 years.

Table I. Age groups and sex distribution in 204 cases under study

S#	Age Groups	Male	Female	Total cases No	%
2	15-25	26	58	84	41 %
3	25-35	27	39	66	32 %
4	35-45	09	24	33	16 %
5	45-55	04	04	08	04 %
6	55-65	06	04	10	05 %
7	65-77	02	01	03	02 %
8	**TOTAL**	**74**	**130**	**204**	**100%**

Discussion

In our study female constitute the major group with 130 (64%) cases while in another study conducted in same area, male preponderance with 54% cases is seen (Sher *et al*., 1996). More frequently involved lymph nodes are that of cervical region with 144 (71%) cases. These results are inline with the results of the study conducted in 2003 by Amin *et al*., with 70% cases of cervical lymph nodes involvement (Amin *et al*., 2003).

The results of our work indicate that benign lymphadenopathy constitutes a significant proportion of findings in aspirates of enlarged lymph nodes with tuberculous lymphadenitis being the main pathological finding followed by the reactive hyperplasia. While in another study, the pattern of incidence of disease was reverse with reactive hyperplasia being the main finding followed by tuberculous lymphadenitis (Pindigo *et al*.

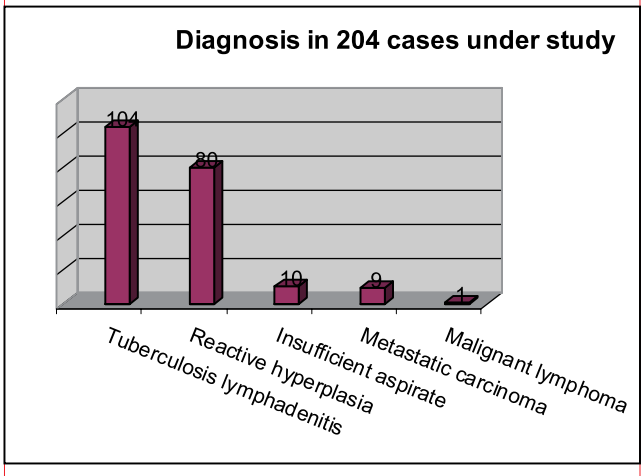

Fig. II. Diagnosis in 204 cases under study

1999). Non-specific reactive lymphadenopathy constituted 80 (39%) cases, which has been documented as a common cause of lymph node enlargement in the tropics (Pindigo *et al.* 1999, Alash *et. al.* 2002). Higher figures have however been observed in children. In this series, cervical lymph nodes (71%) are frequently involved. These figures are in line with reports in the literature (Alash *et al*. 2002)**.**

FNAC was found to be highly effective (94%) in diagnosis and lymph nodes in the posterior triangle of neck were mostly involved (Maharjan *et al*., 2009). Results of our study reveals most commonly involved site is the lymph nodes of cervical region. 71% and 77% cases of reactive hyperplasia and tuberculosis respectively were suffering from cervical lymphadenopathy. Similar findings were published by Abdullah (2000) with 80% involvement of cervical lymph nodes (Abdullah *et al*., 2000).

Total 104 (51%) cases were of tuberculous lymphadenitis in our study. Seventy seven (74%) cases of tuberculous lymphadenitis belong to the age group of 15-35 years, which are the most economically productive age groups of the society**.** In one study the commonest age group affected was 11 – 20 years and constitutional symptoms were not present in most of the patients (Mohapatra and Janmeja , 2009). Tuberculosis has re-emerged as significant public health problem in world and also in Pakistan after an apparent decline in the number of cases some time back. In 2008, globally an estimated 11.1 million people were living with Tuberculosis. Prevalence of tuberculosis in Pakistan is 310/100,000 population while its global prevalence is 170 per 100 000 population. Pakistan is 42 countries with high prevalence of tuberculosis (WHO, 2011)**.** This is an alarming situation as M. tuberculosis which can invades important organs like lungs, bones, intestine, kidney, brain and lymph node affecting their function and quality of patient's life. FNAC of enlarged lymph nodes carries a high diagnostic accuracy. It provides important clues in guiding subsequent clinical management. However, for detailed subtyping of certain disease entities such as lymphoma, surgical biopsy for histological and immunohistochemical studies are required (Sun *et. al.*, 2008)**.** FNAC is a valuable tool and if properly performed and interpreted, than patient can no longer suffer from any delay or misdiagnosis (Ghosh *et al*., 2000). It was observed that the diagnosis of tuberculous lymphadenitis can be made definitely when granulomas composed of epitheloid cells and Langhan's cells are seen. But even in the absence of granulomas, necrosis along with the presence of lymphocytes alone gives an indirect evidence of tuberculous lymphadenitis. However in patients with pyogenic tuberculous lymphadenitis may not necessarily exhibit such a picture. Moreover, FNAC of advanced tuberculous lymphadenitis may frequently display changes that are incompatible with nonspecific-reactive hyperplasia. That is why it is always stressed that in a clinically suspected case, especially if the aspirate contains pus, a bacteriological examination should be tested for acid-fast bacilli and a culture made to improve the diagnostic accuracy. Indeed, the latter was raised to 79% by using the Ziehl Neelsen stain. Same results were found in studies conducted in developing countries (Martelli , 1989). In regions where tuberculosis is endemic, treatment can be instituted without the need for excisional biopsy if the FNA results show characteristic caseating granuloma.

In our study, female were the frequent sufferers of tuberculous lymphadenitis with 67 (64%) cases while 37

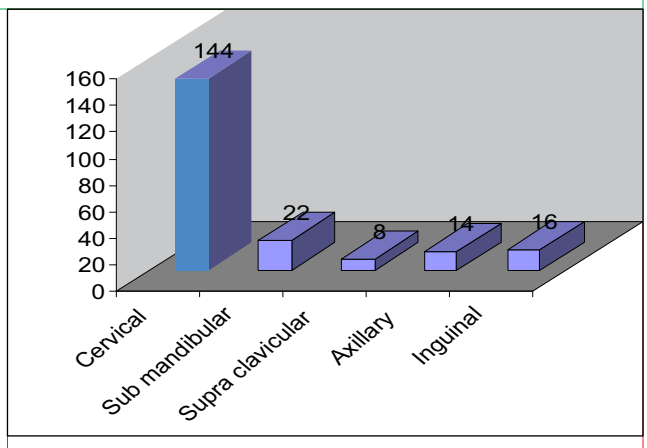

Fig. III. Distribution of the cases under study as per their anatomical site

Table II. Diagnosis according to the anatomical sites of the lymph nodes

Diagnosis	No of Cases	Anatomical Sites of lymph nodes				
		Cervical LN	Sub mandibular LN	Supra Clavicular LN	Axillary LN	Inguinal LN
Insufficient Aspirate	10	03 (1.47)	02 (0.98%)	02(0.98%)	01(0.49%)	02(0.98%)
Reactive hyperplasia	80	57 (27.9%)	14 (6.86%)	02(0.98%)	02(0.98%)	05(2.45%)
Tuberculous lymphadenitis	104	80 (39.2%)	04 (1.96%)	03(1.47%)	09(4.4%)	08 (3.92%)
Metastatic Carcinoma	09	04 (1.96%)	02(0.98%)	01(0.49%)	01(0.49%)	01(0.49%0
Malignant Lymphoma	01	-	-	-	01(0.49%)	0
TOTAL	204	144 (70.5%)	22 (10.8%)	08 (3.9%)	14 (6.9%)	16 (7.9%)

Table III. Lymph node disorders according to age and sex distribution

S #	Diagnosis	Case	%	Male	%	Female	%	M:F	Age range (Years)	Mean age (Years)
1	Insufficient Aspirate	10	4.9	03	1.4	7	3.4	1 : 2.3	15-47	26
2	Reactive hyperplasia	80	39.2	30	14.7	50	24.5	1: 1.6	15-65	32
3	Tuberculous lymphadenitis	104	51.0	37	18.1	67	32.8	1: 1.8	16-85	27
4	Metastatic Carcinoma	09	4.3	04	1.9	05	2.4	1: 1.2	25-45	32
5	Malignant Lymphoma	01	0.5	-	-	01	0.4		15-20	19
6	**Total**	204	100	74	36	130	64	1 :1.7	15-85	27

(36%) male were suffering from this infection. These results have same pattern as that of the study conducted in India in 2009 with 68% female sufferes (Agarwal *et al*., 2009). Same pattern of tuberculous lymphadenitis with these results are in lines with 70% of the female sufferers was found in a study conducted by (Ajmal and Amin, 2003). This sex dependant trend is probably due to the social behavior in Pakistan where female of any age group are more malnourished than male and if they become sick their treatment is not considered the first hand priority as compare to the male member of the family. These discriminative social behaviors can be more frequently observed in the rural population than the urban one.

In our study 3 (1%) cases of malignant lymphoma were also diagnosed on the basis of FNAC. For the diagnosis of lymphoma, FNA provides excellent cytomorphologic material if adequately sampled. The evaluation of FNA in patients with no previously diagnosed malignancy, or in those with suspected lymphoma, should be performed with extreme caution, taking care to obtain a clinical correlation and a confirmatory tissue biopsy, especially in cytologically suspicious cases. However, if malignancy has been previously diagnosed, the legitimate clinical utilization of FNA does not always require follow-up by open biopsy. False-positive results should therefore be reduced to a minimum, since a positive cytologic diagnosis often supports important management decisions (Singh *et al*., 1999). On the other hand, false-negative cases tend to be more common, and are generally based on sampling rather than diagnostic errors, such as the absence of Reed-Sternberg cells, which are important in the diagnosis of Hodgkin disease. However, low-grade (well differentiated) lymphomas with minimal cytomorphologic atypia remained very difficult to evaluate cytologically. Some authors still find it difficult to resolve with certainty the differential diagnosis of lymphoma from reactive hyperplasia or even granulomatous lymphadenitis. Therefore, consultation between cytopathologists and clinicians is mandatory and may result in repeating aspiration or recommending a surgical biopsy. In malignant lymphadenopathies, this inexpensive, relatively painless and rapid technique may not only help in the primary diagnosis of tumors, but remains a useful method of following up patients with known malignancies, and even guiding therapy.

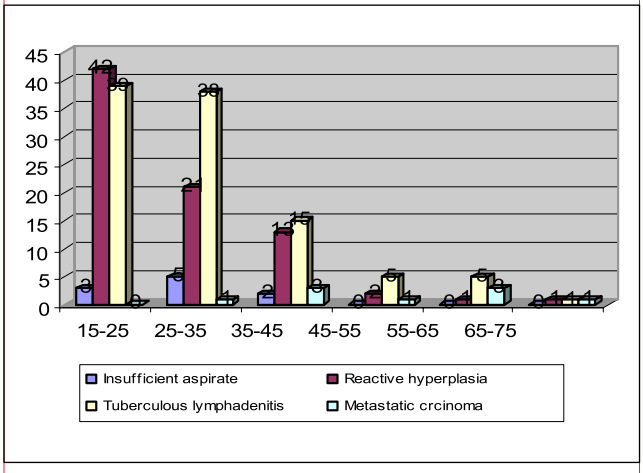

Fig. IV. Age distribution of patients with the four major specific causes of lymphadenopathy

It is concluded that the pattern of disease in this study was similar to other countries of region. Tuberculosis and reactive hyperplasia were the major pathologies and presented mostly with cervical lymphadenopathy. It is also concluded that FNA is the integral tool for the initial diagnosis and management of patients presenting with lymphadenopathy since it offers a high degree of accuracy, lending itself to outpatient diagnosis. Finally this cost-effective and minimally invasive technique can be applied to assess any accessible lesion with existing facilities in Pakistan.

REFERENCES

1. Abdullah P, Mubarak A and Zahir N. 2000. The importance of lymph node biopsy in diagnosis of lymphadenopathy. Journal of College of Physicians and Surgeons Pakistan. 10(8): 298-301.
2. Afridi SP, Beg MA, Memon F. Presentation of enlarged lymph nodes. 2005. Journal of Surgery Pakistan.10:41-3.
3. Agarwal AK, Sethi A, Sethi D, Malhotra V, Singal S. 2009. Tubercular cervical adenitis: clinicopathologic analysis of 180 cases. Journal of otolaryngology - head & neck surgery. 38:521-5.

4. Ajmal, F, and Amin I. 2003. Comparison of FNAC vs Excision Biopsy for suspected Tuberculous Cervical Lymphadenopathy. Annals of King Edward Medical College. 9(3):216-8.
5. Alash, NI, Majeed A and AlHashimi AS. 2002. Lymphadenopathy: histopathologic study of 1145 biopsies. Journal of the Faculty of Medicine Iraq. 36: 171-9.
6. Amin M, Luqman M, Jamal S, Memoon N and Anwar M. 2003. Fine Needle Aspiration Biopsy of Lymphnodes. Journal of College of Physicians and Surgeons Pakistan. 13(11): 625-628.
7. Ghosh, A, Misra RK, Sharma SP, Singh HN and Chaturvedi AK. 2000. Aspiration vs nonaspiration technique of cytodiagnosis--a critical evaluation in 160cases. Indian Juornal of Pathological Microbiology. 43(2): 107-12.
8. Iraqi Cancer Registry Centre. Results of Iraqi Cancer Registry 1989/1991. Institute of Radiology and Nuclear Medicine, Baghdad, Iraq, 1993.
9. Maharjan M, Hirachan S, Kafle PK, Bista M, Shrestha S, Toran KC, 2009. Incidence of tuberculosis in enlarged neck nodes, our experience. Kathmandu University of Medicine Journal. 7:54-8.
10. Mandong BM, Nwana EJC, Igun G, Dakum AN. 1999. Surgical lymphnode biopsies in Jos University Teaching Hospital, Jos, Nigeria. Nigeria Journal or Surgical Research. 1 (3) : 63-67.
11. Martelli, G. 1989. Fine needle aspiration cytology in superficial lymph nodes. European Journal of Surgical Oncology.15 (2): 13-6.
12. Mohapatra PR, Janmeja AK.2009. Tuberculous lymphadenitis. Journal of Associations of Physicians of India. 57:585-90.
13. Pandit AF, Candes and S. Khubchandani. 1987. Fine needle aspiration cytology of lymph nodes. Journal of postgraduate medicine. 33(3): 134.
14. Pindigo UH, Dogo D, Yawe T. 1999. Histopatholgy of primary peripheral lymphaenopathy in north eastern Nigeria. Nigeria Journal or Surgical Research. 1: 68-71.
15. Sher MS, Ali S and Rathi SL. 1996. Fine needle aspiration cytology of superficial palpable lumps. Pakistan Journal of Medical Research. 35 (2): 98-99.
16. Singh MK, Verma K and Kapila K. 1999. Value of aspiration cytology in diagnosis and subtyping malignant lymphomas. Indian Journal of Medical Research. 87 (2):32-6.
17. Sun HB, Zheng XF, Zhang J. 2008. Diagnostic accuracy of fine needle aspiration biopsy of cervical lymph node: a study of 580 cases. Chinese Journal of Pathology. 37:693-7.
18. www.who.int/tb/data. Accessed on 12th of August 2011.

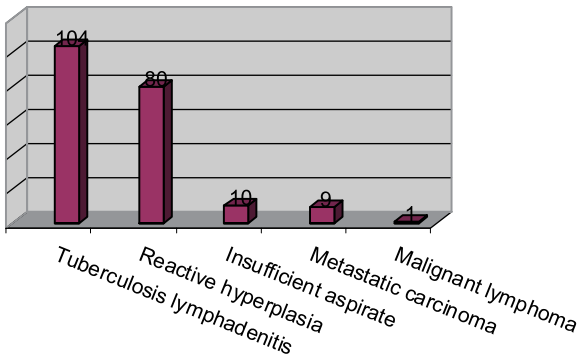

Fig. II. Diagnosis in 204 cases under study

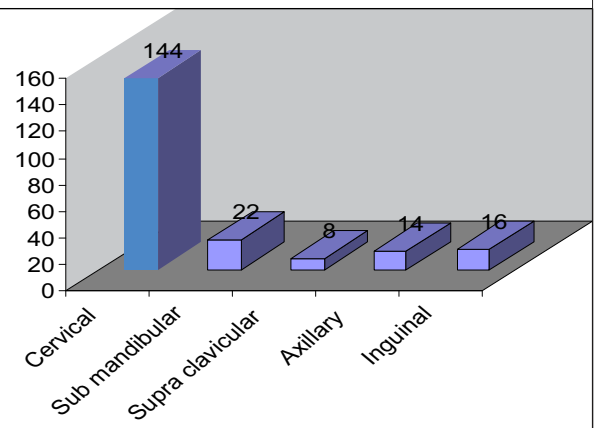

Fig. III. Distribution of the cases under study as per their anatomical site

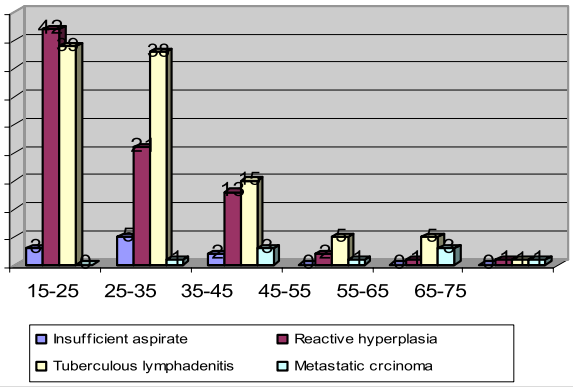

Fig. IV. Age distribution of patients with the four major specific causes of lymphadenopathy

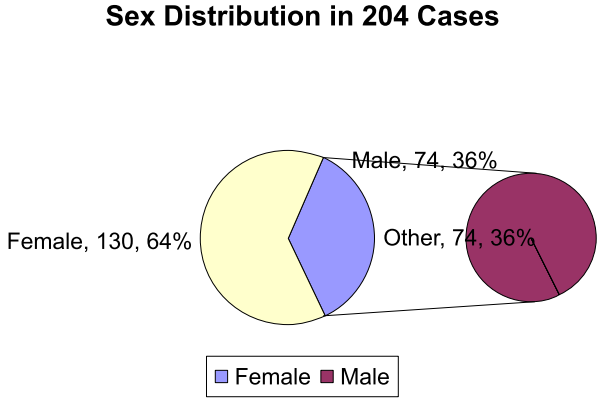

Fig I. Sex Distribution in 204 Cases

Original Article

Amplification and Cloning of Entire Structural Genome (Core-E2) of Hepatitis C Virus

Farakh Javed, Sobia Manzoor, Huma Tariq, Fahed Pervaiz, Muhammad Bilal, Naghmana Kanwal
Atta-ur-Rahman School of Applied Biosciences
lcianunique@yahoo.com

Abstract

HCV is the leading cause of liver related morbidity and mortality around the world. Chronic infection usually leads to serious outcomes like cirrhosis, hepatocellular carcinoma and metabolic abnormalities. Inability of HCV to replicate in cell culture and absence of efficient and cost effective animal models are the major hurdles in developing therapeutic strategies against this virus. Cloning and expression of HCV entire structural genome in eukaryotic expression system was observed in the current study. Structural genes of HCV are important in mediating viral entry in the host cell and pathogenesis, most notably hepatocellular carcinoma. HCV 3a is the most prevalent genotype in Pakistan. RNA was extracted from HCV positive serum infected with 3a genotype, and entire structural genome (C-E2) was reverse transcribed. HCV (C-E2) region was amplified using PCR with gene specific primers having restriction sites. Digested Product of this amplicon was cloned in mammalian expression vector pcDNA3.1+. Positive clones were confirmed after double restriction digestion and sequencing PCR. This successful clone of (C-E2) would be a useful tool for transfection to particular cell lines and further investigation on stable cell lines using this clone may help in designing new therapies and studying interaction of viral proteins with host cells.

Keywords: Hepatitis C Virus, Hepatocellular carcinoma, Polymerase chain reaction, Entire structural genome.

Introduction

Hepatitis C Virus (HCV) is a major human pathogen, affecting about 170 million people in the world (3% of total population). About 350,000 people face death due to HCV-related liver diseases annually (Koziel and Peters, 2007). In Pakistan alone, about 10% of the population is infected chronically with HCV (Idrees and Riazuddin, 2008). Although cleared from the bodies of 20% patients, the virus leads to chronic hepatitis in rest of the patients that ultimately results in cirrhosis, liver failure and hepatocellular carcinoma. A majority of patients also develop metabolic abnormalities like steatosis and insulin resistance (Parvaiz *et al*., 2011; Petta *et al*., 2011). The only line of defense against this devastating virus is a combination therapy of pegylated interferon α and ribavirin, which is not without severe side effects (Zeuzem, 2008). Also, the outcome largely depends on a variety of factors including host immune system, stage of disease and genotype of the virus (El Khattib *et al*., 2012). Lack of efficient and cost effective in vitro models for studying molecular pathways of pathogenesis and screening of candidate antiviral drugs is the major limiting factor in study of HCV (Butt *et al*., 2011; Couto and Kolykhalov, 2006; Tariq *et al*., 2011).

HCV is a member of family Flaviviridae that also includes yellow fever virus, dengue virus, and other viruses that cause diseases in humans and animals (Chambers *et al*., 2003). On the basis of variations in HCV genome, the virus can be classified into six genotypes and numerous subtypes. The viral genome is a positive stranded RNA of 9.6kb that contains a single open reading frame (Kato, 2000). This open reading frame is translated into a single, long polypeptide consisting of 3010 amino acids (Lindenbach and Rice, 2005). The 5' untranslated region of the genome contains an internal ribosome entry site (IRES) that mediates translation of viral genome (Kato *et al*., 2004; Lukavsky, 2009).

Upon processing by viral proteases, this polyprotein is cleaved into 10 functional proteins. The first 3 proteins form structural components (Core, E1, and E2) of mature virus particles. The remaining 7 proteins (viz. p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are non-structural proteins that interact with host proteins in various manners or function as enzymes during virus replication and assembly (Chevaliez and Pawlotsky, 2006). The region enclosing 5' UTR and core gene is mostly conserved while E2/p7 domain contains a "hyper-variable" region that is responsible for resistance to interferon therapy. An alternate reading frame, present in the core gene region, also produces a protein early after the entry of virus in the host cell (Walewski *et al*., 2001). The exact biological role of this protein has been difficult to assess, however, it has been implicated in mitochondrial pathways of apoptosis and production of pro-inflammatory cytokines (Drouin *et al*., 2010).

The HCV core protein forms nucleocapsid that encloses RNA genome of the virus. The core protein has been used as an indicator of HCV infection in ELISA based diagnostic tests (Daniel *et al*., 2007; El Awady *et al*., 2006; Lee *et al*., 2007; Park *et al*., 2010; Reddy, Dakshinamurty, and Lakshmi, 2006). This protein has been shown to play non-structural roles as well by disrupting cell signaling pathways. Transgenic mice expressing core protein alone develop hepatocellular carcinoma (Tanaka *et al*., 2008). Amino acid substitutions in core region also

Corresponding Author: Sobia Manzoor

affect the rate of carcinogenesis (Akuta *et al*., 2011; Kanda *et al*., 2011). Core protein has also been implicated in accumulation of lipid droplets in liver leading to hepatic steatosis (Khan *et al*., 2010). Amino acid substitutions in this protein may predict the outcome of interferon therapy (Kitamura *et al*., 2010; Toyoda *et al*., 2011).

The envelop proteins E1 and E2 are surface glycoproteins associated with lipid membrane surrounding the virus. The C-terminal of both of these proteins contains hydrophobic amino acid residues that anchor the proteins in membrane (Vieyres *et al*., 2010). The N-terminal domains are heavily glycosylated and mediate interaction of viral particle with host cell receptors (Helle *et al*., 2010). These proteins promote fusion of viral and host cell membranes in a pH dependent manner in endosomes (Bartosch, Dubuisson, and Cosset, 2003). This idea is supported by the fact that neutralizing murine antibodies against these proteins block the entry of virus in cells (Bartosch *et al*., 2003; Vieyres, Dubuisson, and Patel, 2011). This finding suggests that envelop proteins are suitable candidate targets for antiviral drugs, that may inhibit the entry of virus in the cells (Liu *et al*., 2010).

The current study focuses on cloning and expression of structural region (core, E1, E2) from local HCV isolates.

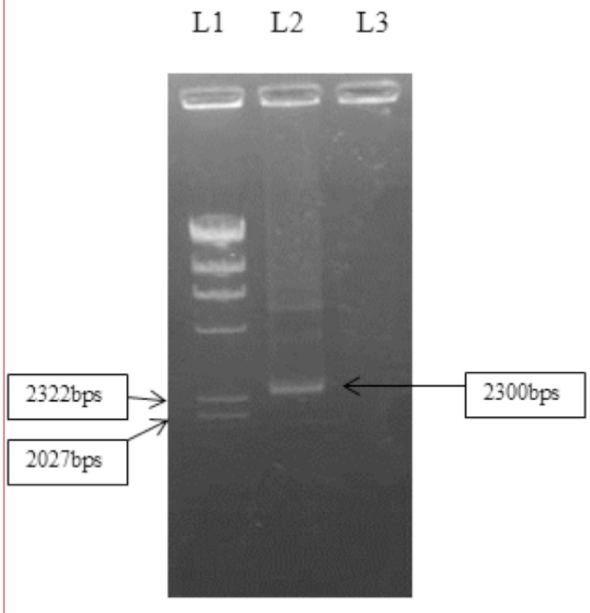

Fig. 3.1: Amplified DNA product of HCV entire structural genome (C-E2). L1 has shown C-E2 region with 2300bps band, L2 has shown Hindi III as a reference while L3 has shown negativecontrol without any amplification.

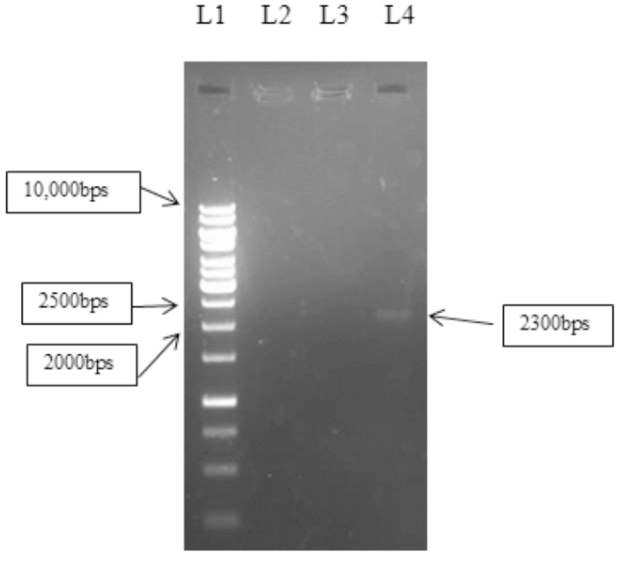

Fig 3.2: Gel purified product of C-E2. L1 has shown 1KB ladder as a reference, L2 & L3 have no product while L4 has shown the purified DNA product with 2300 bps band

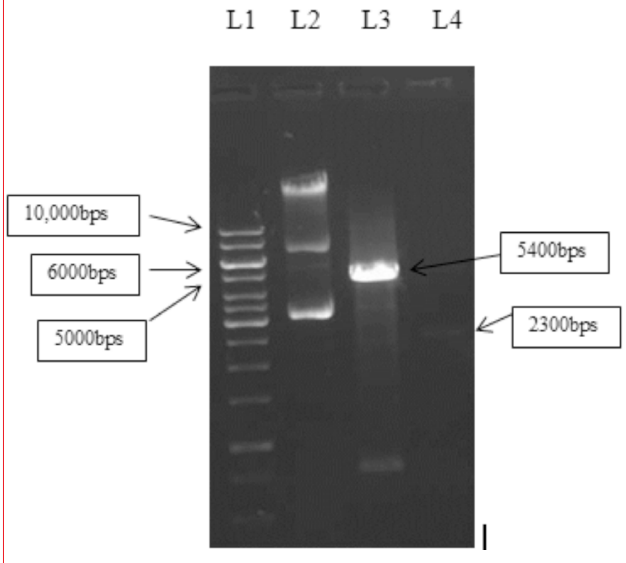

Fig. 3.3: Double digestion of C-E2 DNA product and pcDNA 3.1+ plasmid. L1 has shown 1KB ladder as reference, L2 has shown undigested plasmid, L3 has shown the digested plasmid of with 5.4kb band while L4 has shown digested product of C-E2 region with 2300bps band

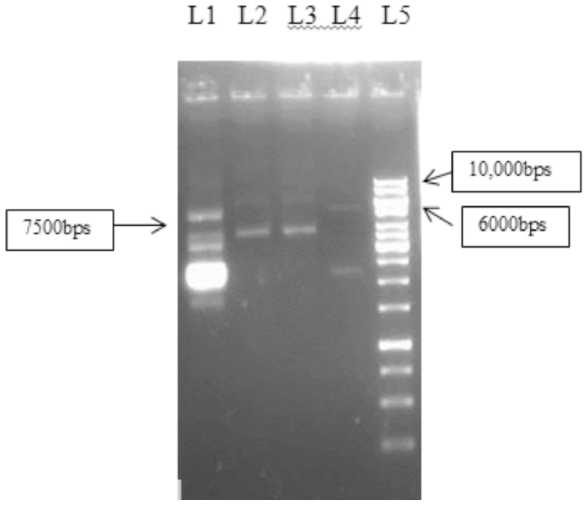

Fig 3.4: Plasmid isolation of four colonies for screening of positive clones of C-E2 in mammalian expression vector pcDNA3.1+. L1-L4 have shown the plasmid isolation of selected colonies while L5 has shown 1KB ladder as reference.

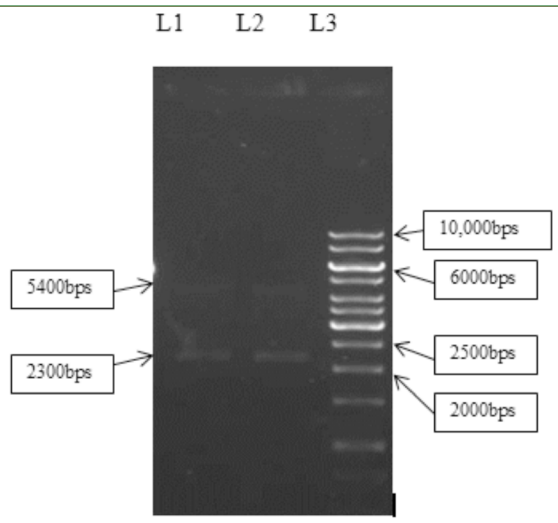

Fig. 3.5: Double restriction digestion of C-E2 clones. L1 and L2 showing the double restriction digestion with 5.4Kb band of plasmid and 2300bps band of C-E2 region, while L3 showing the 1KB ladder as reference.

This may allow investigation of candidate drugs in cell culture model developed at ASAB. Such a system can also help to study molecular pathways perturbed by these proteins in host cells as well as host proteins that interact with them.

Methods

Sample collection

Chronic HCV infected with genotype 3a positive samples were obtained from ASAB Diagnostics (National University of Sciences and Technology) Islamabad, Pakistan.

Polymerase chain reaction of HCV entire structural genome (C-E2)

From HCV positive serum with 3a genotype, RNA was extracted using viral RNA extraction kit (Qiagen). Entire structural genome (C-E2) was reverse transcribed using M-MLV (Fermentas). HCV reference sequence of NZL # D17763 was used for primer designing using primer 3 software.

Entire structural genome (C-E2) was amplified using cocktail of Dream Taq and long pole Taq polymerase (Fermentas) with the following PCR profile i.e. initial denaturation at 95°C for 3minutes (step 1), denaturation at 95°C for 40sec, annealing at 69°C for 40sec, elongation at 68°C for 3mins (step 2 with 35 cycles) and final elongation at 70°C for 10minutes (step 3). Amplified DNA product was observed on 0.8% Agarose gel in UV transilluminator (Wealtec) and was gel purified using silica bead DNA gel extraction kit (Fermentas).

Cloning of HCV entire structural genome into mammalian expression vector

Double digested DNA product of entire structural genome (C-E2) was ligated using T4 DNA ligase (Fermentas) with mammalian expression vector pcDNA 3.1+ which was also digested with same restriction enzymes and transformed into *E.coli* Top-10 competent cells. Successful clone was confirmed by double restriction digestion using FastDigest EcoR1 (Fermentas) and FastDigest BamHI (Fermentas).

Results

PCR of HCV entire structural genome (C-E2)

Entire structural genome (C-E2) was amplified from HCV positive sera sample no 1918. Fig. 3.1 has shown the amplification of C-E2 region with 2300 bps band and Hindi III ladder as a reference on 0.8% Agarose gel. While fig. 3.2 has shown the gel purified product of C-E2 with 2300bps band and 1KB ladder.

Double digestion of PCDNA 3.1+ vector and C-E2 DNA product

Double digestion of C-E2 DNA product and Mammalian expression vector pcDNA 3.1+ was performed using FastDigest EcoR1 and FastDigest BamHI (fig 3.3).

Cloning of HCV entire structural genome into mammalian expression vector

After successful transformation of C-E2 region with pcDNA3.1+ total four colonies were cultured for screening, out of which two colonies have shown the positive clones. Plasmid isolation has shown in fig. 3.4 while double restriction digestion has shown in fig.3.5.4.

Discussion

More than twenty years have passed since the discovery and isolation of hepatitis C virus. Despite tremendous efforts, there is no drug available against this virus except ribavirin, while some drugs are in pre-clinical or clinical phase. This can be attributed to the lack of efficient, reliable, consistent and robust models for the study of HCV life cycle (Couto and Kolykhalov, 2006). Acknowledging the need of time, a vast number of studies are being directed towards achieving successful replication of HCV in cell culture (Tariq *et al*., 2011). Nevertheless, only one HCV isolate, derived from fulminant hepatitis C genotype 2a, has been shown to replicate successfully in cell culture system to date (Wakita and Kato, 2006).

Subtle differences exist in biology and pathology of different genotypes and even subtypes of HCV. The outcome of interferon therapy is also dependent largely on the genotype of the virus (Andriulli *et al*., 2008). These differences are due, in part, to differences in nucleotide and amino acid differences (Donlin *et al*., 2010). Since no full length virus of genotype 2 and 3 has been reported to replicate in cell culture to date, there is a need to resort to alternative strategies to study viral biology and response to novel drugs. Previously, studies have shown to produce stable cell lines expressing parts of HCV genome (Butt *et al*., 2011; Takahashi *et al*., 2005) to study viral protein interactions. HCV subgenomic replicons have been produced to study viral replication and regulation of its life cycle (Arai *et al*., 2011; Blight and Norgard, 2006; Flint *et al*., 2009; Howe *et al*., 2008).

The current study successfully cloned and expressed HCV structural proteins of genotype 3a in cell line. Establishment of this cell line may prove instrumental in study of HCV replication and virus-host interactions. This will allow the investigation of viral and cellular factors involved in entry and infection of virus particles in the cells. In future, such systems may help in large-scale screening and testing of novel antiviral compounds against HCV proteins.

Conclusion

HCV entire structural genome (Core-E2) was successfully cloned in the mammalian expression system pcDNA3.1+ which will help to observe HCV structural genes mediated modulation of gene expression in stably expressing transfected cells.

REFRENCES

1. Akuta, N., Suzuki, F., Hirakawa, M., Kawamura, Y., Sezaki, H., Suzuki, Y., Hosaka, T., Kobayashi, M., Saitoh, S., Arase, Y., Ikeda, K., and Kumada, H. 2011. Amino acid substitutions in hepatitis C virus core region predict hepatocarcinogenesis following eradication of HCV RNA by antiviral therapy. J Med Virol 83(6):1016-1022.
2. Andriulli, A., Mangia, A., Iacobellis, A., Ippolito, A., Leandro, G., and Zeuzem, S. 2008. Meta-analysis: the outcome of anti-viral therapy in HCV genotype 2 and genotype 3 infected patients with chronic hepatitis. Aliment Pharmacol Ther 28(4):397-404.
3. Arai, M., Suzuki, H., Tobita, Y., Takagi, A., Okamoto, K., Ohta, A., Sudoh, M., and Kohara, M. 2011. Establishment of infectious HCV virion-producing cells with newly designed full-genome replicon RNA. Arch Virol 156(2):295-304.
4. Bartosch, B., Bukh, J., Meunier, J.C., Granier, C., Engle, R.E., Blackwelder, W.C., Emerson, S.U., Cosset, F.L., and Purcell, R.H. 2003a. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. Proc Natl Acad Sci U S A 100(24):14199-14204.
5. Bartosch, B., Dubuisson, J., and Cosset, F.L. 2003b. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med 197(5):633-642.
6. Blight, K.J., and Norgard, E.A. 2006. HCV Replicon Systems.
7. Butt, S., Idrees, M., Rehman, I.U., Ali, L., Hussain, A., Ali, M., Ahmed, N., Saleem, S., and Fayyaz, M. 2011. Establishment of stable Huh-7 cell lines expressing various hepatitis C virus genotype 3a protein: an in-vitro testing system for novel anti-HCV drugs. Genet Vaccines Ther 9(1):12.
8. Chambers, T.J., Maramorosch, K., Shatkin, A.J., Monath, T.P., and Murphy, F.A. 2003. The Flaviviruses: Structure, Replication and Evolution. Elsevier.
9. Chevaliez, S., and Pawlotsky, J.M. 2006. HCV Genome and Life Cycle. In: Tan SL, editor. Hepatitis C viruses: genomes and molecular biology: Horizon Bioscience.
10. Couto, L.B., and Kolykhalov, A.A. 2006. Animal Models for HCV Study.
11. Donlin, M.J., Cannon, N.A., Aurora, R., Li, J., Wahed, A.S., Di Bisceglie, A.M., and Tavis, J.E. 2010. Contribution of genome-wide HCV genetic differences to outcome of interferon-based therapy in Caucasian American and African American patients. PLoS One 5(2):e9032.
12. Drouin, C., Lamarche, S., Bruneau, J., Soudeyns, H., and Shoukry, N.H. 2010. Cell-mediated immune responses directed against hepatitis C virus (HCV) alternate reading frame protein (ARFP) are undetectable during acute infection. J Clin Virol 47(1):102-103.
13. El Khattib, A.A., Abdelhakam, S.M., Ghoraba, D.M., Ibrahim, W.A., and Sayed, M.M. 2012. Outcome of antiviral therapy in Egyptian Hepatitis C Virus (HCV) genotype 4 patients with advanced liver fibrosis. Eur J Intern Med 23(1):e34-35.
14. Flint, M., Mullen, S., Deatly, A.M., Chen, W., Miller, L.Z., Ralston, R., Broom, C., Emini, E.A., and Howe, A.Y. 2009. Selection and characterization of hepatitis C virus replicons dually resistant to the polymerase and protease inhibitors HCV-796 and boceprevir (SCH 503034). Antimicrob Agents Chemother 53(2):401-411.
15. Helle, F., Vieyres, G., Elkrief, L., Popescu, C.I., Wychowski, C., Descamps, V., Castelain, S., Roingeard, P., Duverlie, G., and Dubuisson, J. 2010. Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. J Virol 84(22):11905-11915.
16. Howe, A.Y., Cheng, H., Johann, S., Mullen, S., Chunduru, S.K., Young, D.C., Bard, J., Chopra, R., Krishnamurthy, G., Mansour, T., and O'Connell, J. 2008. Molecular mechanism of hepatitis C virus replicon variants with reduced susceptibility to a benzofuran inhibitor, HCV-796. Antimicrob Agents Chemother 52(9):3327-3338.
17. Idrees, M., and Riazuddin, S. 2008. Frequency distribution of hepatitis C virus genotypes in different geographical regions of Pakistan and their possible routes of transmission. BMC Infect Dis 8:69.
18. Kanda, T., Imazeki, F., Yonemitsu, Y., Mikami, S., Takada, N., Nishino, T., Takashi, M., Tsubota, A., Kato, K., Sugiura, N., Tawada, A., Wu, S., Tanaka, T., Nakamoto, S., Mikata, R., Tada, M., Chiba, T., Kurihara, T., Arai, M., Fujiwara, K., Kanai, F., and Yokosuka, O. 2011. Quantification of hepatitis C virus in patients treated with peginterferon-alfa 2a plus ribavirin treatment by COBAS TaqMan HCV test. J Viral Hepat 18(7):e292-297.
19. Kato, J., Shiratori, Y., Kato, N., and Omata, M. 2004. [Structure and function of the internal ribosome entry site of HCV]. Nihon Rinsho 62 Suppl 7(Pt 1):38-42.
20. Kato, N. 2000. Genome of human hepatitis C virus (HCV): gene organization, sequence diversity, and variation. Microb Comp Genomics 5(3):129-151.

21. Khan, M., Jahan, S., Khaliq, S., Ijaz, B., Ahmad, W., Samreen, B., and Hassan, S. 2010. Interaction of the hepatitis C virus (HCV) core with cellular genes in the development of HCV-induced steatosis. Arch Virol 155(11):1735-1753.
22. Kitamura, S., Tsuge, M., Hatakeyama, T., Abe, H., Imamura, M., Mori, N., Saneto, H., Kawaoka, T., Mitsui, F., Hiraga, N., Takaki, S., Kawakami, Y., Aikata, H., Takahashi, S., Ohishi, W., Ochi, H., Hayes, C.N., and Chayama, K. 2010. Amino acid substitutions in core and NS5A regions of the HCV genome can predict virological decrease with pegylated interferon plus ribavirin therapy. Antivir Ther 15(8):1087-1097.
23. Koziel, M.J., and Peters, M.G. 2007. Viral hepatitis in HIV infection. N Engl J Med 356(14):1445-1454.
24. Lindenbach, B.D., and Rice, C.M. 2005. Unravelling hepatitis C virus replication from genome to function. Nature 436(7053):933-938.
25. Liu, R., Tewari, M., Kong, R., Zhang, R., Ingravallo, P., and Ralston, R. 2010. A peptide derived from hepatitis C virus E2 envelope protein inhibits a post-binding step in HCV entry. Antiviral Res 86(2):172-179.
26. Lukavsky, P.J. 2009. Structure and function of HCV IRES domains. Virus Res 139(2):166-171.
27. Parvaiz, F., Manzoor, S., Tariq, H., Javed, F., Fatima, K., and Qadri, I. 2011. Hepatitis C virus infection: molecular pathways to insulin resistance. Virol J 8:474.
28. Petta, S., Camma, C., Di Marco, V., Macaluso, F.S., Maida, M., Pizzolanti, G., Belmonte, B., Cabibi, D., Di Stefano, R., Ferraro, D., Guarnotta, C., Venezia, G., and Craxi, A. 2011. Hepatic steatosis and insulin resistance are associated with severe fibrosis in patients with chronic hepatitis caused by HBV or HCV infection. Liver Int 31(4):507-515.
29. Takahashi, H., Yamaji, M., Hosaka, M., Kishine, H., Hijikata, M., and Shimotohno, K. 2005. Analysis of the 5' end structure of HCV subgenomic RNA replicated in a Huh7 cell line. Intervirology 48(2-3):104-111.
30. Tanaka, N., Moriya, K., Kiyosawa, K., Koike, K., and Aoyama, T. 2008. Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor alpha in transgenic mice: implications for HCV-associated hepatocarcinogenesis. Int J Cancer 122(1):124-131.
31. Tariq, H., Manzoor, S., Parvaiz, F., Javed, F., Fatima, K., and Qadri, I. 2011. An overview: In vitro models of HCV replication in different cell cultures. Infect Genet Evol.
32. Toyoda, H., Kumada, T., Kaneoka, Y., and Maeda, A. 2011. Amino acid substitutions in the hepatitis C virus core region are associated with postoperative recurrence and survival of patients with HCV genotype 1b-associated hepatocellular carcinoma. Ann Surg 254(2):326-332.
33. Vieyres, G., Dubuisson, J., and Patel, A.H. 2011. Characterization of antibody-mediated neutralization directed against the hypervariable region 1 of hepatitis C virus E2 glycoprotein. J Gen Virol 92(Pt 3):494-506.
34. Vieyres, G., Thomas, X., Descamps, V., Duverlie, G., Patel, A.H., and Dubuisson, J. 2010. Characterization of the envelope glycoproteins associated with infectious hepatitis C virus. J Virol 84(19):10159-10168.
35. Wakita, T., and Kato, T. 2006. Development of an Infectious HCV Cell Culture System.
36. Walewski, J.L., Keller, T.R., Stump, D.D., and Branch, A.D. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. RNA 7(5):710-721.
37. Zeuzem, S. 2008. Interferon-based therapy for chronic hepatitis C: current and future perspectives. Nat Clin Pract Gastroenterol Hepatol 5(11):610-622.

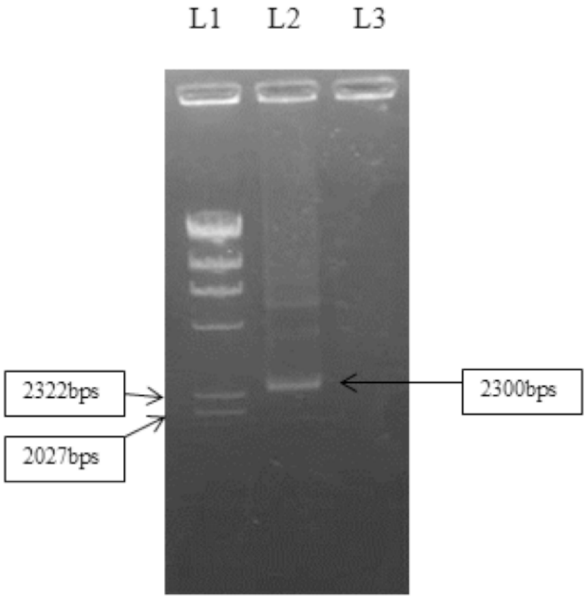

Fig. 3.1: Amplified DNA product of HCV entire structural genome (C-E2). L1 has shown C-E2 region with 2300bps band, L2 have shown Hindi III as a reference while L3 shown negative control without any amplification.

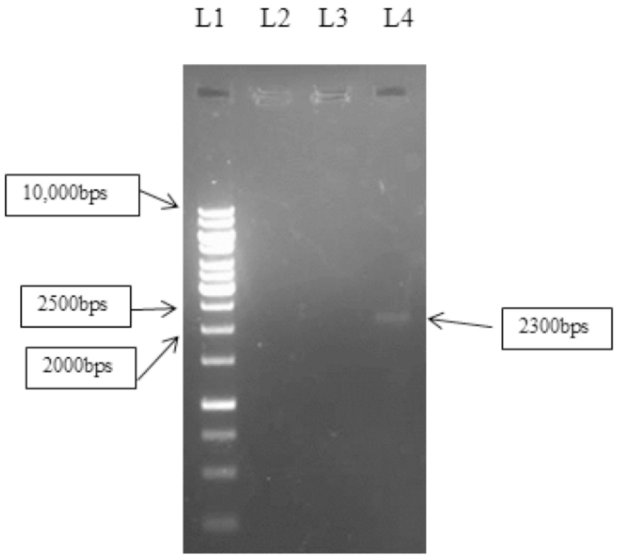

Fig 3.2: Gel purified product of C-E2. L1 have shown 1KB ladder as a reference, L2 & L3 has no product while L4 shown the purified DNA product with 2300 bps band

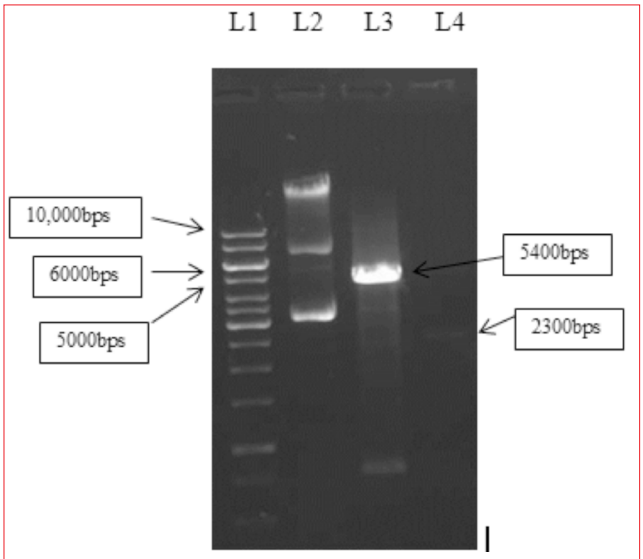

Fig. 3.3: Double digestion of C-E2 DNA product and pcDNA 3.1+ plasmid. L1 has shown 1KB ladder as reference, L2 shown undigested plasmid, L3 has shown the digested plasmid of with 5.4kb band while L4 has shown digested product of C-E2 region with 2300bps band

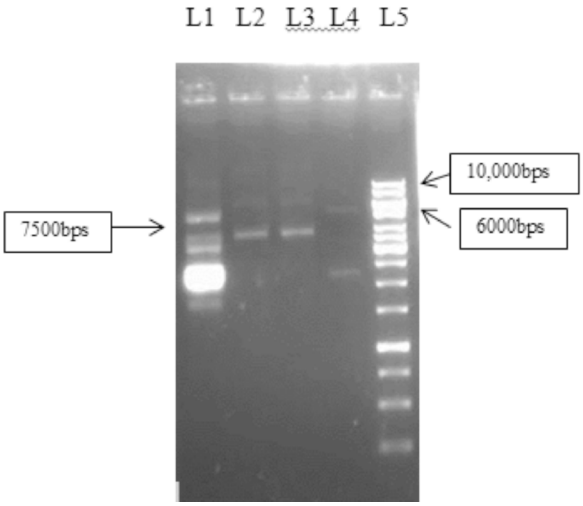

Fig 3.4: Plasmid isolation of four colonies for screening of positive clones of C-E2 in mammalian expression vector pcDNA3.1+. L1-L4 has shown the plasmid isolation of selected colonies while L5 has shown 1KB ladder as reference.

Review Article

An update on the pathophysiology and pharmacology of Alzheimer' disease

Touqeer Ahmed
Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology
touqeer.aahmed@gmail.com

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease. Plaques and tangles are described as the characteristics features of AD, which are neurotoxic entities, deposited in the diseased patients. There are very few drugs available for the treatment of AD. Those available options are primarily the approaches to overcome cholinergic hypofunction, through the inhibition of acetylcholinesterase enzyme. Other disease modifying candidates are strongly needed to overcome the progressive dementia in AD. These new drugs will serve as a great hope for the AD patients and families of the AD patients.

Introduction

Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia which was explained by a German psychiatrist Dr. Alois Alzheimer (1864-1915) for the first time in 1906. This disease is known by the name of Dr. Alzheimer as "Alzheimer's disease" and this name was given by Kraeplin (Blennow et al., 2006). Dr. Alzheimer came across a patient during his experience and later he described his first case, representing as a true picture of AD with plaques and fibrils bundled as neurofibrillary tangles (Selkoe, 2001, Blennow et al., 2006, Avramopoulos, 2009). These plaques and tangles are neurotoxic in nature (Ahmed et al., 2011). Today it has been understood that the AD is a chronic progressive neurodegenerative disease and it is the most common form of dementia, accounting for 50–60 % of all cases (Blennow et al., 2006). There is a profound cholinergic hypofucntion in AD, which results in decline in the cognitive functions (Ahmed and Gilani, 2009, Ahmed et al., 2010). Clinical symptoms in AD can be sub-divided into three groups such as; (i) cognitive dysfunction (memory loss, language difficulties and intellectual function), (ii) psychiatric symptoms which include agitation, depression, delusion, hallucination, insomnia and wandering (Burns et al., 1990, Lahiri. et al., 2002, Burns and Iliffe, 2009) and collectively these are grouped as non-cognitive symptoms and (iii) group of symptoms represented by the AD patients, such as difficulties in performing activities of daily life, including shopping, eating, dressing and driving (Lahiri. et al., 2002, Burns and Iliffe, 2009). AD progresses from memory loss to the substantial dementia and death within eight years of the age (Avramopoulos, 2009).

Epidemiology and Risk Factors

Prevalence

AD has been established to be one of the most common forms of dementia. It is estimated that half of the people over the age of 85 are affected with AD (Suh and Checler, 2002), affecting about 10 % population of the world (Vagnucci and Li, 2003). The prevalence of the dementia below 60-64 years of the aged population is 1 %, but increases exponentially with increasing age and at the age of 85 years and older, prevalence is found to be 25–33 % in western countries (Ferri et al., 2005, Blennow et al., 2006). AD represents as the sixth leading cause of deaths and these figures are rising. In 2001, 24 million people were reported suffering from dementia and this figure is going to be doubled every 20 years, with expected number of around 81 million cases in 2040 (Ferri et al., 2005). Representing data from the developing countries is sparse but estimates show that 60 % of the AD patients are expected to live developing countries (Blennow et al., 2006). There is limited data available regarding the prevalence of AD in Pakistan because of the limited reports from Pakistani population, but the figures from China (neighbour country) indicate high prevalence like western countries (Zhang et al., 2005). According to a recent estimate, over 5.3 million people in USA alone have AD, with 5.1 million people over the age of 65 years and 0.2 million below 65 years of age. In terms of its economic impact, estimated direct and indirect cost of AD is USD 148 billion annually.

Risk factors

Aging represents as the most established risk factor (Lahiri. et al., 2002, Ferri et al., 2005). Epidemiological studies have also established association of several risk factors with AD. Low educational and occupational level, low mental ability during early life and reduced activity in late life to be associated with AD (Mayeux, 2003, Mortimer et al., 2003). Several studies have shown as head injury to be one of the risk factor in AD (Kalaria, 2001, Marx, 2001, Lahiri. et al., 2002, Jellinger, 2004). There has also been evidence for the role of cardiovascular health in AD (Mayeux, 2003).

Genetics of Alzheimer's disease

Like the classical case represented by the Alois Alzheimer,

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the onset of this devastating disorder is relatively not in very old age population, mean age is below 65 years of the age (given the name of pre-senile dementia). This lead scientists to distinguish this from senile dementia and soon led to the discovery of genes involved in familial, autosomal dominant AD (FAD). First mutation to be identified was in amyloid precursor protein (APP) located on chromosome 21 (Goate et al., 1991). Moreover, additional mutation were found in presenilin 1 (*PSEN1* located on chromosome 14) and presenilin 2 (*PSEN2* located on chromosome 1) which account for most of the cases of familial AD (Levy-Lahad et al., 1995a, Levy-Lahad et al., 1995b, Sherrington et al., 1995). Especially, when it comes to the late onset of the disease the genetics are much more complicated (Avramopoulos, 2009) and *APOE* (located on chromosome 19) was found to be associated with AD (Corder et al., 1993, Poirier et al., 1993), where, it poses as a risk factor in late AD (Strittmatter et al., 1993, Burns and Iliffe, 2009). APOE has three isoforms and APOE ϵ4 is responsible for modifying the onset of AD (Poirier et al., 1993) with increasing risk several times in APOE ϵ4 allele homozygotes (Farrer et al., 1997). Each allele copy lowering the age by almost 10 years (Corder et al., 1993) and accounts for most of the sporadic cases (Raber et al., 2004). Until now several mutations have been identified and show association, whose details could be found at "www.alzgene.org".

Pathogenesis of Alzheimer's disease

AD is characterized by the deposition of the amyloid plaques (also known as senile plaques which are composed of the amyloid peptides (Aβ)) located extracellularly and neurofibrillary tangles (intracellular). Neurofibrillary tangles are composed of hyperphoshorylated tau proteins (Selkoe, 2001, Palmer, 2002) that has detrimental effect on synapses and neurons, resulting in the degenerative changes, by the activation of inflammatory pathways, oxidative stress and mitochondrial dysfunction (Kroemer et al., 2007). Amyloid plaques originate from amyloid precursor protein (APP) which is type I transmembrane glycoprotein (Suh and Checler, 2002), processed by various proteases generating Aβ fragment. APP is processed either through amyloidogenic or non-amyloidogenic pathway.

Under the normal conditions when Aβ peptide is produced, it is cleared by the process of degradation through various peptidases, such as, insulin degrading enzyme, neprilysin, and by endothelin converting enzyme (Carson and Turner, 2002). The famous amyloid cascade hypothesis explains that in AD, balance between the production and effective clearance is lost with dominating production, thus resulting in the deposition of Aβ peptide and leading to the development of the dementia (Hardy and Selkoe, 2002).

On the other hand tangles in the brain are composed of hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986). Tau is a key protein that binds microtubules through its microtubules binding domain and provides the basis of microtubules functional assembly into the elongated structure in the axons. In the normal state tau is phosphorylated (by GSK-3β, CDK5) and dephosphorylated (by PP-1 and PP-2A) and there is balance between these two processes (Iqbal et al., 2005). But in AD this balance is towards the hyperphosphorylation of the tau proteins, thus microtubules subunits loose assembly and fall apart, disrupting axonal structure as well as the normal transport, this event results in severely compromised synaptic function (Iqbal et al., 2005).

In addition to these pathologies there is an initial selected loss of the cholinergic neurons in basal forebrain whose axons project into cortex and hippocampus (Fisher, 2000). As a result of this loss, there is decrease in intellectual function specially defect in memory. Cholinergic hypothesis provided the basis for the development of therapy in AD which provides symptomatic treatment.

Mechanism of cell death in Alzheimer's disease

Different mechanisms of neuronal cell death have been manifested in AD. Aβ is a protein fragment which is cleaved product of the APP. Aβ is neurotoxic which deposits in the brain of AD patients. Aβ deposition activates different signaling pathways which are responsible for cell death. Various mechanisms are proposed, which account for the Aβ induced toxicity. It has been reported that Aβ induces toxicity by the enhancement of Ca^{++} channels (Mattson et al., 1992), which results in the rise in intracellular Ca^{++} through voltage dependent Ca^{++} channels (Davidson et al., 1994, Weiss et al., 1994). Ca^{++} has various functions in cell signaling when in physiological concentration. Rise in intracellular Ca^{++} beyond certain limits can be lethal for cells, leading to cytotoxicity. In addition to Ca^{++} influx, Aβ fragments elevate glutamate release (Harkany et al., 2000). Electrophysiological studies in cortical pyramidal neurons indicate that Aβ induces excitatory post synaptic potential and train of action potentials by increasing excitability of glutamatergic projections (Gu et al., 2003), end result of this is again rise in intracellular cations specially Ca^{++}. Intracellular rise in Ca^{++} burden directs cells for apoptosis.

Beta amyloid fragments in the primary hippocampal neurons have shown to induce apoptosis through the mitochondrial pathways (Nilsen et al., 2006), thus translocating Bax to mitochondria resulting in release of the cytochrome C and activating apoptotic pathway.

Currently available treatment options

Drachman and Leavitt reported in 1974 that muscarinic antagonists, such as scopolamine produces cognition impairment similar to memory deficit observed in elderly people (Drachman and Leavitt, 1974). This provided a base that cholinergic system has important physiological role in the cognitive functions. Subsequent reports have shown reduction in cholineacetyltransferase activity in cerebral cortex and nucleus basalis of Meynert of patients affected by AD (Liberini et al., 1996). It is now quite evident that degeneration of the basal forebrain neurons as well as loss of cholinergic projections to various cortical areas is

responsible for deficits in neuropsychology (Liberini et al., 1996).

There are two ways to enhance cholinergic functions; one is to inhibit the enzyme acetylcholinesterase (AChE) , responsible for the breakdown of endogenous acetylcholine (ACh) while the other approach is to directly stimulate muscarinic receptors (using muscarinic agonists).

Acetylcholinesterase inhibitors. ACh is an important physiological neurotransmitter, which is quickly hydrolyzed by endogenous enzyme, AChE. The inhibition of the enzyme results in more ACh available to interact with postsynaptic muscarinic receptors. Currently available medicines for AD therapy are AChE inhibitors (Lopez et al., 2002). AChE inhibitor drugs approved by FDA in USA for the symptomatic treatment of AD are are rivastigmine, galantamine and donepezil (Lahiri. et al., 2002, Blennow et al., 2006), which show multiple side-effects limiting their usefulness in AD. Considering the mechanism of AChE inhibition, these drugs provide effective symptomatic treatment for variable period of time (Courtney et al., 2004, Bullock and Dengiz, 2005, Bullock et al., 2005), but do not change the course of AD (Blennow et al., 2006).

Muscarinic agonists. Selective loss of cholinergic neurons in central nervous system served as a target for the development of medicines which can enhance cholinergic transmission for the AD therapy. Selective muscarinic receptor (M_1) agonist is the suitable candidate (Fisher, 2000). M_1 receptors can be targeted because this is the major subtype of muscarinic receptors found in hippocampus and cortex (Friedman, 2004); this strategy can reduce side-effects associated with the stimulation of other muscarinic receptors. The activation of the muscarinic receptors modifies the APP processing and inhibits Aβ production (Gu et al., 2003). Currently, there is limited availability of M_1 agonists which have limitation either due to narrow selectivity resulting in side effects, poor bioavailability or penetration into blood brain barrier, thus requiring larger doses (Fisher, 2000).

Memantine. Memantine is another drug which is non-competitive NMDA receptor antagonist that has protective effect from increased glutamate levels mediated excitotoxicity, without interfering with physiological activation of NMDA receptors (Wilcock, 2003). Clinical trial suggest modest efficacy of memantine in moderate to severe AD patients (Wilcock, 2003).

Potential treatment options highlighted through epidemiology studies

As the knowledge about disease progresses, different pharmacological treatment options are coming into consideration. Several epidemiological studies have shown protective effects of the different drugs; some of them are summarized here.

Anti-inflammatory drugs: AD is also considered as a chronic inflammatory disease (Ahmed and Gilani, 2011). There are several studies supporting the use of anti-inflammatory drugs, with some beneficial effects in AD. Retrospective studies, that compared the frequency of nonsteroidal anti-inflammatory drugs (NSAIDs) use and AD progression, suggested slowing down the onset of AD (Li et al., 1992, Breitner et al., 1994). Ibuprofen, a well known NSAID, exerts beneficial effects by reducing Aβ amyloid deposition and senile plaque formation (Lim et al., 2000). In addition, studies on animals provide consolidation of this concept, that NSAIDs may protect against AD by suppressing inflammatory process (Netland et al., 1998, Lim et al., 2000).

Antioxidants: It has been widely supported in the literature that Aβ fragments produce oxidative radicals (Behl et al., 1994, Butterfield et al., 1994), which play an important role in AD pathogenesis. Therefore, therapeutic interventions to reduce oxidative damage induced injury, may retard and slow down the onset of disease. In-vivo studies as well as cell culture system have shown that Aβ-induced neurotoxicity is attenuated with vitamin E (Behl et al., 1992, Yamada et al., 1999, Huang et al., 2000). Antioxidant, such as Vitamin E intake can reduce the risk of AD (Sano et al., 1997, Engelhart et al., 2002, Morris et al., 2002).

Calcium channel blockers. Ca^{++} channel blockers (CCB) produce their beneficial effects by inhibiting pathological rise in intracellular Ca^{++}, which is the result of different events taking place in AD. In-vitro, Aβ has been shown to form Ca^{++} channels in membranes (Lin et al., 1999), thus, resulting in potentiation of toxicity (Rovira et al., 2002). Nimodipine, a CCB showed attenuation of Aβ-induced toxicity in cell culture (Weiss et al., 1994). In a recent report, CCBs have been shown to have beneficial effects in AD (Vagnucci and Li, 2003). Elderly patients who were treated with CCBs, showed decline in dementia (Forette et al., 1998).

Cholesterol lowering drugs. A few studies have shown to reduce the incidence of disease with usage of cholesterol lowering drugs, such as, statins (Jick et al., 2000, Wolozin et al., 2000), however; evidence becomes weak where some reports either do not show protective effect (Rea et al., 2005, Zandi et al., 2005) or marginal effect (Sparks et al., 2005) with cholesterol lowering drugs.

Future drug targets in Alzheimer's disease with disease modifying potential

The precise cause of the AD is not clear yet. There are series of questions which are still to be answered, such as; plaques come first or the initial selective cholinergic hypofunction; targeting disease by inhibiting plaques and tangles formation or to inhibit the secretase enzyme, or to clear Aβ using vaccines as pharmacological tool.

There are a limited number of medicines available for the treatment of AD, main drugs are AChE inhibitors, and there is a wide room available for newer drugs. AChE inhibitors partly overcome memory deficit in disease providing symptomatic treatment, but do not delay the time course of the disease. Hence, there is a need to combat this

disease by discovering candidates with disease modifying potential. Moreover, targeting through multiple pathways, which are activated as a result of cascades that are responsible to damage neurons need to be targeted. Drugs targeting α, β and γ secretase (enzymes responsible for the processing of APP) activity are being widely explored as target in AD with different degrees of success (Luo et al., 2001, Petit et al., 2001, Chang et al., 2004, Etcheberrigaray et al., 2004, Siemers et al., 2005). In addition, an approach to develop Aβ vaccine is also being investigated (Schenk et al., 1999, Bard et al., 2000). There is also some developing interest to have drugs chelating metal ions, which induce Aβ aggregations and show toxic effects (Cherny et al., 2001, Ritchie et al., 2003)

REFERENCES

1. Alzheimer's Association 2009. pp 1-12.
2. Ahmed T, Enam SA, Gilani AH. 2012. Curcuminoids enhance memory in an amyloid-infused rat model of Alzheimer's disease. Neuroscience, 169:1296-1306.
3. Ahmed T, Gilani AH. 2009. Inhibitory effect of curcuminoids on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia may explain medicinal use of turmeric in Alzheimer's disease. Pharmacol Biochem Behav, 91:554-559.
4. Ahmed T, Gilani AH. 2011. A comparative study of curcuminoids to measure their effect on inflammatory and apoptotic gene expression in an Abeta plus ibotenic acid-infused rat model of Alzheimer's disease. Brain Res, 1400:1-18.
5. Ahmed T, Gilani AH, Hosseinmardi N, Semnanian S, Enam SA, Fathollahi Y. 2011. Curcuminoids rescue long-term potentiation impaired by amyloid peptide in rat hippocampal slices. Synapse, 65:572-582.
6. Avramopoulos D. 2009. Genetics of Alzheimer's disease: recent advances. Genome Med, 1:34.
7. Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Lieberburg I, Motter R, Nguyen M, Soriano F, Vasquez N, Weiss K, Welch B, Seubert P, Schenk D, Yednock T. 2000. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat Med, 6:916-919.
8. Behl C, Davis J, Cole GM, Schubert D. 1992. Vitamin E protects nerve cells from amyloid beta protein toxicity. Biochem Biophys Res Commun, 186:944-950.
9. Behl C, Davis JB, Lesley R, Schubert D. 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell, 77:817-827.
10. Blennow K, de Leon MJ, Zetterberg H. 2006. Alzheimer's disease. Lancet, 368:387-403.
11. Breitner JC, Gau BA, Welsh KA, Plassman BL, McDonald WM, Helms MJ, Anthony JC. 1994. Inverse association of anti-inflammatory treatments and Alzheimer's disease: initial results of a co-twin control study. Neurology, 44:227-232.
12. Bullock R, Dengiz A. 2005. Cognitive performance in patients with Alzheimer's disease receiving cholinesterase inhibitors for up to 5 years. Int J Clin Pract, 59:817-822.
13. Bullock R, Touchon J, Bergman H, Gambina G, He Y, Rapatz G, Nagel J, Lane R. 2005. Rivastigmine and donepezil treatment in moderate to moderately-severe Alzheimer's disease over a 2-year period. Curr Med Res Opin, 21:1317-1327.
14. Burns A, Iliffe S. 2009b. Alzheimer's disease. Bmj, 338:158.
15. Burns A, Jacoby R, Levy R. 1990. Psychiatric phenomena in Alzheimer's disease. IV: Disorders of behaviour. Br J Psychiatry, 157:86-94.
16. Butterfield DA, Hensley K, Harris M, Mattson M, Carney J. 1994. beta-Amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease. Biochem Biophys Res Commun, 200:710-715.
17. Carson JA, Turner AJ. 2002. Beta-amyloid catabolism: roles for neprilysin (NEP) and other metallopeptidases? J Neurochem, 81:1-8.
18. Chang WP, Koelsch G, Wong S, Downs D, Da H, Weerasena V, Gordon B, Devasamudram T, Bilcer G, Ghosh AK, Tang J. 2004. In vivo inhibition of Abeta production by memapsin 2 (beta-secretase) inhibitors. J Neurochem, 89:1409-1416.
19. Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, Barnham KJ, Volitakis I, Fraser FW, Kim Y, Huang X, Goldstein LE, Moir RD, Lim JT, Beyreuther K, Zheng H, Tanzi RE, Masters CL, Bush AI. 2001. Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. Neuron, 30:665-676.
20. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA. 1993. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science, 261:921-923.
21. Courtney C, Farrell D, Gray R, Hills R, Lynch L, Sellwood E, Edwards S, Hardyman W, Raftery J, Crome P, Lendon C, Shaw H, Bentham P. 2004. Long-term donepezil treatment in 565 patients with Alzheimer's disease (AD2000): randomised double-blind trial. Lancet, 363:2105-2115.
22. Davidson RM, Shajenko L, Donta TS. 1994. Amyloid beta-peptide (A beta P) potentiates a nimodipine-sensitive L-type barium conductance in N1E-115 neuroblastoma cells. Brain Res, 643:324-327.
23. Drachman DA, Leavitt J. 1974. Human memory and the cholinergic system. A relationship to aging? Arch Neurol, 30:113-121.
24. Engelhart MJ, Geerlings MI, Ruitenberg A, van Swieten JC, Hofman A, Witteman JC, Breteler MM. 2002. Dietary intake of antioxidants and risk of Alzheimer disease. Jama, 287:3223-3229.
25. Etcheberrigaray R, Tan M, Dewachter I, Kuiperi C, Van der Auwera I, Wera S, Qiao L, Bank B, Nelson TJ, Kozikowski AP, Van Leuven F, Alkon DL. 2004. Therapeutic effects of PKC activators in Alzheimer's

disease transgenic mice. Proc Natl Acad Sci U S A, 101:11141-11146.
26. Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM. 1997. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. Jama, 278:1349-1356.
27. Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y, Jorm A, Mathers C, Menezes PR, Rimmer E, Scazufca M. 2000. Global prevalence of dementia: a Delphi consensus study. Lancet, 366:2112-2117.
28. Fisher A. 2000. Therapeutic strategies in Alzheimer's disease: M1 muscarinic agonists. Jpn J Pharmacol, 84:101-112.
29. Forette F, Seux ML, Staessen JA, Thijs L, Birkenhager WH, Babarskiene MR, Babeanu S, Bossini A, Gil-Extremera B, Girerd X, Laks T, Lilov E, Moisseyev V, Tuomilehto J, Vanhanen H, Webster J, Yodfat Y, Fagard R. 1998. Prevention of dementia in randomised double-blind placebo-controlled Systolic Hypertension in Europe (Syst-Eur) trial. Lancet, 352:1347-1351.
30. Friedman JI.2004. Cholinergic targets for cognitive enhancement in schizophrenia: focus on cholinesterase inhibitors and muscarinic agonists. Psychopharmacology (Berl), 174:45-53.
31. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature, 349:704-706.
32. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI. 1986. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U S A, 83:4913-4917.
33. Gu Z, Zhong P, Yan Z. 2003. Activation of muscarinic receptors inhibits beta-amyloid peptide-induced signaling in cortical slices. J Biol Chem, 278:17546-17556.
34. Hardy J, Selkoe DJ. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science, 297:353-356.
35. Harkany T, Abraham I, Timmerman W, Laskay G, Toth B, Sasvari M, Konya C, Sebens JB, Korf J, Nyakas C, Zarandi M, Soos K, Penke B, Luiten PG. 2000. beta-amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis. Eur J Neurosci, 12:2735-2745.
36. Huang HM, Ou HC, Hsieh SJ. 2000. Antioxidants prevent amyloid peptide-induced apoptosis and alteration of calcium homeostasis in cultured cortical neurons. Life Sci, 66:1879-1892.
37. Iqbal K, Alonso Adel C, Chen S, Chohan MO, El-Akkad E, Gong CX, Khatoon S, Li B, Liu F, Rahman A, Tanimukai H, Grundke-Iqbal I. 2005. Tau pathology in Alzheimer disease and other tauopathies. Biochim Biophys Acta, 1739:198-210.
38. Jellinger KA. 2004. Head injury and dementia. Curr Opin Neurol, 17:719-723.
39. Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA. 2000. Statins and the risk of dementia. Lancet, 356:1627-1631.
40. Kalaria RN. 2001. Advances in molecular genetics and pathology of cerebrovascular disorders. Trends Neurosci, 24:392-400.
41. Kroemer G, Galluzzi L, Brenner C. 2007. Mitochondrial membrane permeabilization in cell death. Physiol Rev, 87:99-163.
42. Lahiri. DK, Farlow. MR, Greig. NH, Sambamurti. K. 2002. Current drug targets for Alzheimer's disease treatment. Drug Development Research, 56:267-281.
43. Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu CE, Jondro PD, Schmidt SD, Wang K, et al. 1995a. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science, 269:973-977.
44. Levy-Lahad E, Wijsman EM, Nemens E, Anderson L, Goddard KA, Weber JL, Bird TD, Schellenberg GD. 1995b. A familial Alzheimer's disease locus on chromosome 1. Science, 269:970-973.
45. Li G, Shen YC, Li YT, Chen CH, Zhau YW, Silverman JM.1992. A case-control study of Alzheimer's disease in China. Neurology, 42:1481-1488.
46. Liberini P, Valerio A, Memo M, Spano PF. 1996. Lewy-body dementia and responsiveness to cholinesterase inhibitors: a paradigm for heterogeneity of Alzheimer's disease? Trends Pharmacol Sci, 17:155-160.
47. Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, Cole GM. 2000. Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. J Neurosci, 20:5709-5714.
48. Lin H, Zhu YJ, Lal R. 1999. Amyloid beta protein (1-40) forms calcium-permeable, Zn2+-sensitive channel in reconstituted lipid vesicles. Biochemistry, 38:11189-11196.
49. Lopez S, Bastida J, Viladomat F, Codina C. 2002. Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and Narcissus extracts. Life Sci, 71:2521-2529.
50. Luo Y, Bolon B, Kahn S, Bennett BD, Babu-Khan S, Denis P, Fan W, Kha H, Zhang J, Gong Y, Martin L, Louis JC, Yan Q, Richards WG, Citron M, Vassar R. 2001. Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. Nat Neurosci, 4:231-232.
51. Marx J. 2001. Alzheimer's disease. Bad for the heart, bad for the mind? Science, 294:508-509.
52. Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. 1992. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci, 12:376-389.
53. Mayeux R. 2003. Epidemiology of neurodegeneration. Annu Rev Neurosci, 26:81-104.

54. Morris MC, Evans DA, Bienias JL, Tangney CC, Bennett DA, Aggarwal N, Wilson RS, Scherr PA. 2002. Dietary intake of antioxidant nutrients and the risk of incident Alzheimer disease in a biracial community study. Jama, 287:3230-3237.
55. Mortimer JA, Snowdon DA, Markesbery WR. 2003. Head circumference, education and risk of dementia: findings from the Nun Study. J Clin Exp Neuropsychol, 25:671-679.
56. Netland EE, Newton JL, Majocha RE, Tate BA. 1998. Indomethacin reverses the microglial response to amyloid beta-protein. Neurobiol Aging, 19:201-204.
57. Nilsen J, Chen S, Irwin RW, Iwamoto S, Brinton RD. 2006. Estrogen protects neuronal cells from amyloid beta-induced apoptosis via regulation of mitochondrial proteins and function. BMC Neurosci, 7:74.
58. Palmer AM. 2002. Pharmacotherapy for Alzheimer's disease: progress and prospects. Trends Pharmacol Sci, 23:426-433.
59. Petit A, Bihel F, Alves da Costa C, Pourquie O, Checler F, Kraus JL. 2001. New protease inhibitors prevent gamma-secretase-mediated production of Abeta40/42 without affecting Notch cleavage. Nat Cell Biol, 3:507-511.
60. Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S. 1993. Apolipoprotein E polymorphism and Alzheimer's disease. Lancet, 342:697-699.
61. Raber J, Huang Y, Ashford JW. 2004. ApoE genotype accounts for the vast majority of AD risk and AD pathology. Neurobiol Aging, 25:641-650.
62. Rea TD, Breitner JC, Psaty BM, Fitzpatrick AL, Lopez OL, Newman AB, Hazzard WR, Zandi PP, Burke GL, Lyketsos CG, Bernick C, Kuller LH. 2005. Statin use and the risk of incident dementia: the Cardiovascular Health Study. Arch Neurol, 62:1047-1051.
63. Ritchie CW, Bush AI, Mackinnon A, Macfarlane S, Mastwyk M, MacGregor L, Kiers L, Cherny R, Li QX, Tammer A, Carrington D, Mavros C, Volitakis I, Xilinas M, Ames D, Davis S, Beyreuther K, Tanzi RE, Masters CL. 2003. Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Abeta amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial. Arch Neurol, 60:1685-1691.
64. Rovira C, Arbez N, Mariani J. 2002. Abeta(25-35) and Abeta(1-40) act on different calcium channels in CA1 hippocampal neurons. Biochem Biophys Res Commun, 296:1317-1321.
65. Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, Woodbury P, Growdon J, Cotman CW, Pfeiffer E, Schneider LS, Thal LJ. 1997. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. N Engl J Med, 336:1216-1222.
66. Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P. 1999. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature, 400:173-177.
67. Selkoe DJ. 2001. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev, 81:741-766.
68. Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, et al. 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature, 375:754-760.
69. Siemers E, Skinner M, Dean RA, Gonzales C, Satterwhite J, Farlow M, Ness D, May PC. 2005. Safety, tolerability, and changes in amyloid beta concentrations after administration of a gamma-secretase inhibitor in volunteers. Clin Neuropharmacol, 28:126-132.
70. Sparks DL, Sabbagh MN, Connor DJ, Lopez J, Launer LJ, Browne P, Wasser D, Johnson-Traver S, Lochhead J, Ziolwolski C. 2005. Atorvastatin for the treatment of mild to moderate Alzheimer disease: preliminary results. Arch Neurol, 62:753-757.
71. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD. 1993. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci U S A, 90:1977-1981.
72. Suh YH, Checler F. 2002. Amyloid precursor protein, presenilins, and alpha-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer's disease. Pharmacol Rev, 54:469-525.
73. Vagnucci AH, Jr., Li WW. 2003. Alzheimer's disease and angiogenesis. Lancet, 361:605-608.
74. Weiss JH, Pike CJ, Cotman CW. 1994. Ca2+ channel blockers attenuate beta-amyloid peptide toxicity to cortical neurons in culture. J Neurochem, 62:372-375.
75. Wilcock GK. 2003. Memantine for the treatment of dementia. Lancet Neurol, 2:503-505.
76. Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G. 2000. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. Arch Neurol, 57:1439-1443.
77. Yamada K, Tanaka T, Han D, Senzaki K, Kameyama T, Nabeshima T. 1999. Protective effects of idebenone and alpha-tocopherol on beta-amyloid-(1-42)-induced learning and memory deficits in rats: implication of oxidative stress in beta-amyloid-induced neurotoxicity in vivo. Eur J Neurosci, 11:83-90.
78. Zandi PP, Sparks DL, Khachaturian AS, Tschanz J, Norton M, Steinberg M, Welsh-Bohmer KA, Breitner JC. 2005. Do statins reduce risk of incident dementia and Alzheimer disease? The Cache County Study. Arch Gen Psychiatry, 62:217-224.
79. Zhang ZX, Zahner GE, Roman GC, Liu J, Hong Z, Qu QM, Liu XH, Zhang XJ, Zhou B, Wu CB, Tang MN, Hong X, Li H. 2005. Dementia subtypes in China: prevalence in Beijing, Xian, Shanghai, and Chengdu. Arch Neurol, 62:447-453.

Review Article

An Overview of Herbal Antiviral Compounds Against Dengue Virus (Ae. aygepti)"

Sobia Tabassum*, Faiza Abdul Rashid, Hina Javed, Muhammad Ashraf**
*Institution: Biotechnology and Bioinformatics, H-10 Campus, International Islamic University, Islamabad
**Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology (NUST) H-12 Islamabad, Pakistan.
sobia.tabasum@iiu.edu.pk

Abstract

Dengue is the current prevalent disease caused by vector *A. aegypti* having four serotypes DENV 1,2,3,4. Natural biological systems are disrupted by the repeated use of synthetic repellents and often results in the development of resistance against dengue virus. These problems have emphasized the need for the development of new strategies for the selective control of mosquito larvae. Therefore antiviral active compounds are extracted from natural herbs to treat dengue virus. The aim of this paper is to review some opportunities in the development of anti-dengue drugs from celery, black seed, *ponneem, Cucurbitaceae U. tomentosa, Gastrodiaelata Bl, L. alba* and *L. citriodora, Vitextrifolia* and citrus plants like lemon, orange which might be helpful to control the epidemic.

Keywords : Dengue, A. aegypti, Antiviral, Drugs , Black seed, Ponneem.

Introduction

Transmission of malaria, dengue fever, yellow fever, filariasis, schistosomiasis, and Japanese encephalitis are caused by major vector mosquitoes (James 1992; Gubler 1998). Among these dengue is the current prevalent disease. According to World Health Organization (WHO 2003), around 50 million dengue virus cases per year are estimated (Valdéz *et al*. 2009). Aedes mosquitoes, humans and lower primates are three natural hosts for dengue virus.

Characteristics of Dengue Virus

Dengue virus is associated with the Flaviviridae family and has four serotypes DENV: 1, 2, 3, and 4. Dengue haemorrhagic fever and dengue shock syndrome are two severe forms of dengue fever (Hendarto and Hadinegoro 1992, Pancharoen *et al*. 2002). Virion consists of single-stranded RNA molecule of approximately 11 kb in length. The viral genome of dengue virus consist of three structural proteins, capsid (C), pre-membrane (prM), envelope (E) and seven non-structural proteins (NS1, **A**
2009; Talarico *et al*. 2007). The role of E protein is to interact within the virus (Rice 2007). Aedes aegypti mosquito is a vector for the transmission of DENV. Less effective transmitters are *Ae. albopictus and Ae. polynesiensis* (Ooi *et al*. 2009).

Control of Vectors Through Insecticides

Applications of organophosphates and insect growth regulators are more frequently used to control the mosquito larvae (Yang *et al*. 2002). Insecticides and many synthetic agents have been developed for the control of mosquito-borne diseases. The disadvantage of using insecticides is that they are non-selective and are harmful to other organisms also. Natural biological systems are disrupted by the repeated use of synthetic repellents and often results in the development of resistance (Rozendaal 1997), provoke undesirable effects including toxicity to non-target organisms (Lee *et al*. 2001), this lead to the need for novel insecticides (Macedo *et al*. 1997).

The Need to Screen Antiviral Compounds from Natural Herbs

These problems have emphasized the need for the development of new strategies for the selective control of mosquito larvae. Till now there is no antiviral drug for the treatment of Flavivirus and no vaccine is yet available. Therefore the development of antiviral drugs is required to prevent dengue mortalities. Traditional medicinal plants and herbs consist of active compounds that have antiviral activity (Jassim & Naji 2003). From various herb species oils are extracted that can directly inactivate the virus. These antiviral compounds possess unique biological activity and can act aslarvicides, insect growth regulators, repellents, and have restrictive activities (Mathivanan *et al*. 2010; Niraimathi *et al*. 2010; Samidurai *et al*. 2009).

There are many natural herbs which show antiviral activity against dengue virus. Drugs can be designed by using these natural herbs, potency of some of them are discussed here.

Antiviral Activity of Para-Benzoquinones

Unsubstituted para-benzoquinone (with no alkyl group). Greater the methyl groups attached to the ring, greater will be the potency of para-benzoquinone (table 1). By appropriate structural modification of para-benzoquinones, it may be possible to develop novel insecticidal compounds potentially suitable to control the dengue mosquito (Damiao *et al*. 2010).

Antiviral Activity of Cucurbitaceae Plants

Solvent extracts of five species of *Cucurbitaceae* plants show great larvicidal activity (figure 1). The petroleum ether extracts of *C. colocynthis*, methanol extracts of *C. indica*, *C. sativus*, *M. charantia,* and acetone extract of *T. anguina* are effective against dengue virus (Rahman *et al*. 2008)

Corresponding Author: Sobia Tabassum

Table 1 Larvicidal activities (LC_{50}) and 95% CI of evaluated compounds 1–6 on third-instar larvae of *A. aegypti*

Compound	LC_{50} ppm (CI)
1	90 (70 to 107)
2	61 (50 to 76)
3	33 (25 to 44)
4	42 (34 to 51)
5	57 (44 to 72)
6	48 (40 to 56)
Temephos	0.042 (0.035 to 0.05)

The antiviral and immune modulating effects of u. Tomentosa

U. tomentosa pentacyclicoxindole alkaloids displayed the antiviral and immune-modulating *in vitro* effects. Due to this nature novel properties are explored for the therapy of Dengue Fever (Sonia *et al*. 2008).

Anti-Dengue Virus Bioactivities of Gastrodiaelata Bl Plant

Two alpha-D-glucans WGEW and AGEW from Gastrodiaelata Bl show anti-dengue virus bioactivities. There structures were explained by using gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). The deduced structures are alpha-D-(1--> 4)-glucan with an alpha (1-- >4) linked branch attached to O-6 branch points with dissimilar branch degrees. Distinct degrees of substitution (DS) were prepared by adding sulphate derivatives. Strong anti-dengue virus bioactivities are showed by all sulfated derivatives.

Antiviral property of L. Alba and L. Citriodora oil

L. alba and *L. citriodora* oil has inhibitory effect on all four DENV serotypes. The essential oil penetrates within the skin and produce inhibitory effect to block viral replication (Carson *et al*. 2001). Plaque reduction assay is performed to examine antiviral activities of the selected essential oils. Against all examined viruses 50% inhibition of plaque formation was observed (figure 2, 3).

Antiviral Activity of Citrus Plants against Dengue Virus

Citrus seeds and peel of citrus plants have been tested against insects and proved to be effective. The extracts of *Citrus grandis* (Chakutra), *Citrus paradisi* (Grape Fruit), *Citrus jambhiri* (rouph lemon) and *Citrus reticulata* (Kinnow) are more effective against dengue virus infections (Waseem *et al*. 2010).

Antviral Activity of Vitextrifolia Leaves against Ae. Agypti

From the methanol extract of *Vitextrifolia* leaves, a crystalline compound methyl-*p*-hydroxybenzoate is isolated and structure is elucidated by NMR and single crystal X-ray diffractometer (Figure 4). This compound

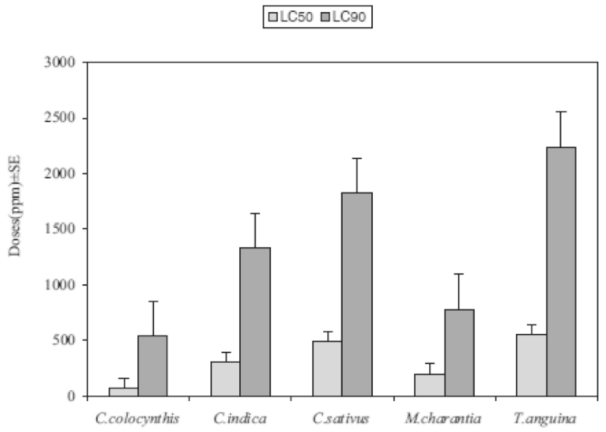

Fig. 1. Antiviral activity of five species of cucurbitaceae plant

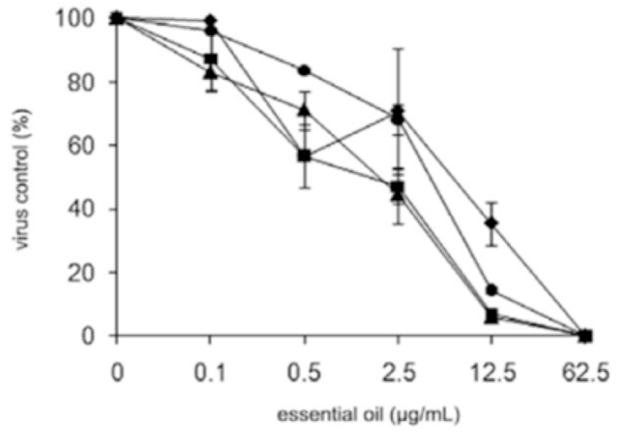

Fig 2. Lippia alba essential oil effects on plaque formation of DENV, DENV2, DENV3, DENV

possesses 100% larval mortality of *Ae. aygepti* (Kannathasan *et al*. 2011).

Ponneem A Herbal Formulation against Dengue Virus

Oils of neem (*Azadirachtaindica*) and karanj (*Pongamiaglabra*) forms a novel herbal formulation known as PONNEEM. Active compounds of *A.indicaareazadirachtin, salanin, nimbidin, nimbin, nimbolide, mahmoodin and geduninandin P. glabra, karanjin are* oleic acid, linoleic acid, linolenic acid, palmitic acid and stearic acid (Maheswaran *et al*. 2011).

Nematocidal activity of celery against ae. Aegypti

Bioactive compounds of *Apiumgraveolens* (Celery) possess nematocidal activity against *Ae. Aegypti* (Rafikali *et al*. 2000; Rafikali and Muraleednaran 2001).

Black Seed a Valuable Remedy of Dengue Infection

Black seed, *Nigella sativa* belongs to Ranunculaseae family. It has active compounds like Quinones, thymoquinones, dithymoquinones (Daba and Abdel-

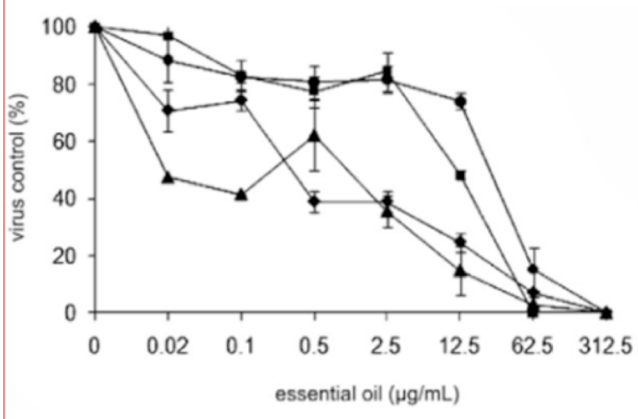

Fig. 3. Lippiacitriodoraessential oil effects on plaque formation of DENV 1, DENV2, DENV3, DENV4

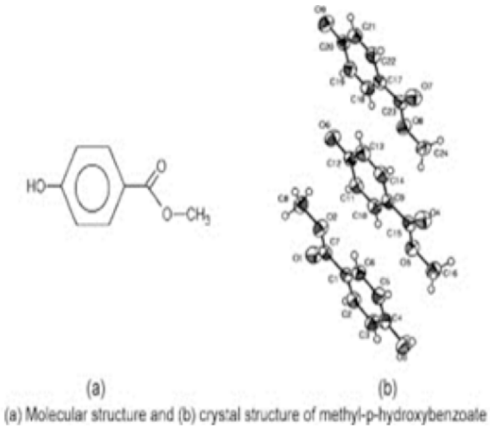

Fig.4. Molecular and crystal structure of methyl-p-hydroxy benzoate.

Rehman, 1998, Naji *et al* 1999). Thymoquinones carries antiviral activity against *Ae. agypti* by enhancing the immune response of vertebrates (Ahmed *et al*. 2008)

Novel anti-dengue compounds *from Rhizophoraapiculata Blume*, *Piper retrofractum Vahl*, *Flagellariaindica Linn*, *Cladogynosorientalis Zipp* and *Houttuyniacordata Thunb* have not yet been identified. In future there will be a need to focus on the purification, exact mechanism of their antiviral action, and characterization of their active compounds.

It is concluded from the present review that the, natural herbs possess lead compounds for the development of larvicidal activity. These compounds could be investigated in detail with the objective of isolation and characterization of biologically active molecules which could be used as lead compounds in drug discovery.

REFERENCES

1. Abdul Rahuman A and Venkatesan P 2008. Larvicidal efficacy of five cucurbitaceous plant leaf extracts against mosquito species. Parasitology Research, 103:133–139.
2. Cheng S, Huang C, Chen W, Kuo Y, Cheng S 2008. Larvicidal activity of tectoquinone isolated from red heartwood-type Cryptomeria japonica against two mosquito species. Bioresource Technology, 99:3617–3622.
3. Gubler DJ. 1998. Resurgent vector borne diseases as a global health problem. Emergence of Infectious Diseases, 4:442–450.
4. Hendarto SK and Hadinegoro SR. 1992. Dengue encephalopathy. Acta Paediatrica Japonica, 34:350-357.
5. James AA. 1992. Mosquito molecular genetics: the hands that feed bite back. Science, 257:37–38.
6. Jassim S and Naji M. 2003. Novel antiviral agents: a medicinal plant perspective. Journal of Applied Microbiology, 95: 317-412.
7. Lee SE, Kim JE and Lee HS. 2001. Insecticide resistance in increasing interest. Agricultural Chemistry & Biotechnology, 44:105–112.
8. Lindenbach B, Rice C. 2007. Flaviviridae: the viruses and their replication. In D Knipe D, P Howley, *Fields virology*, Willians& Wilkins, Philadelphia, pp. 991-1041.
9. Macedo ME, Consoli RAGB, Grandi TSM, Anjos AMG, Oliveira AB, Mendes NM, Queiroz RO and Zani CL. 1997. Screening of Asteraceae (Compositae) plant extracts for larvicidal activity against *Aedesfluviatilis* (Diptera: Culicidae). Memórias do Instituto Oswaldo Cruz, 92:565–570.
10. Ooi EE and Gubler DJ 2009. Dengue in Southeast Asia: epidemiological characteristics and strategic challenges in disease prevention. Cadernos de Saúde Pública, 25:115-24.
11. Pancharoen C, Kulwichit W, Tantawichien T, Thisyakorn U and Thisyakorn C. 2002. Dengue infection: a global concern. Journal of Medical Association of Thiland, 85:25-33.
12. Rothwell C, Lebreton A and Chuan Young Ng, *et al* 2009. Cholesterol biosynthesis modulation regulates dengue viral replication. Virology, 389:8-19.
13. Rozendaal JA 1997. Mosquitoes and other biting Diptera. In: Vector control. World Health Organization, Geneva, Switzerland, pp. 5–177.
14. Sánchez-Valdéz E, Delgado AM, Torres-Martínez JA, Torres-Benítez JM 2009. Clinical response in patients with dengue fever to oral calcium plus vitamin D administration: study of 5 cases. Proceedings of the Western Pharmacology Society, 52:14–17.
15. Talarico LB and Damonte EB 2007. Interference in dengue virus adsorption and uncoating by carageenans. Virology, 363:473-85.
16. Yang YC, Lee SG, Lee HK, Kim MK, Lee SH and Lee HS. 2002. A piperidine amide extracted from *Piper longum L.* fruit shows activity against *Aedesaegypti* mosquito larvae. Journal of Agricultural and Food Chemistry, 50:3765–3767.

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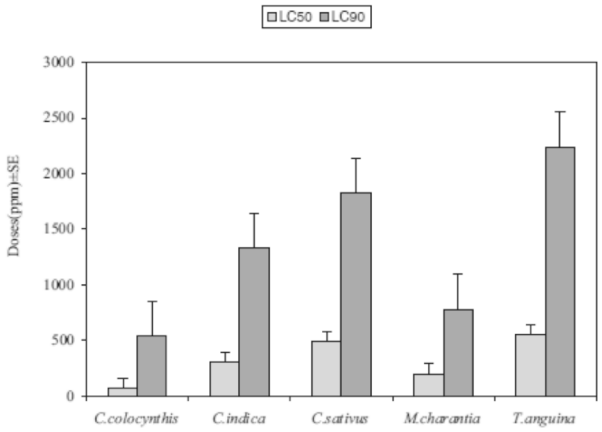

Fig. 1. Antiviral activity of five species of cucurbitaceae plant

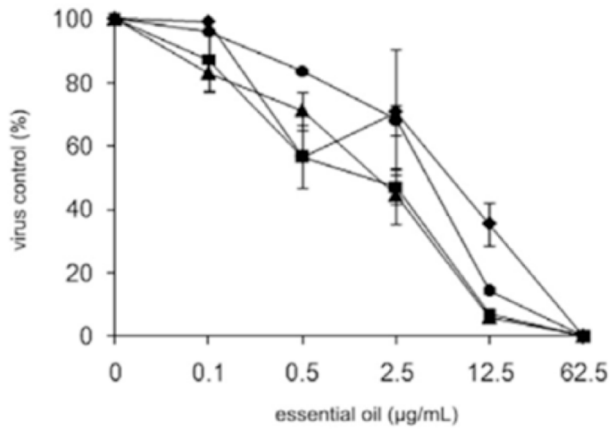

Fig 2. Lippia alba essential oil effects on plaque formation of DENV , DENV2 , DENV3, DENV

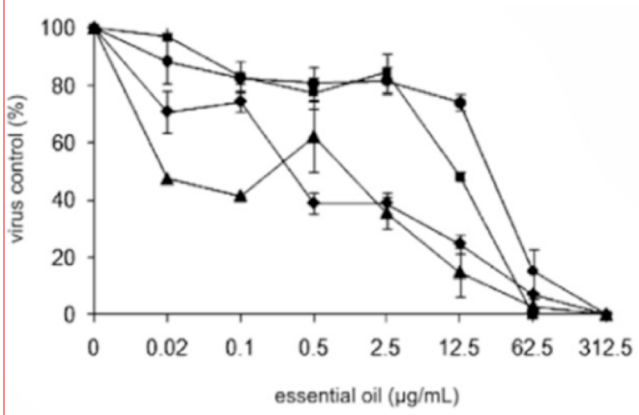

Fig. 3. Lippiacitriodoraessential oil effects on plaque formation of DENV 1 ,DENV2, DENV3, DENV4

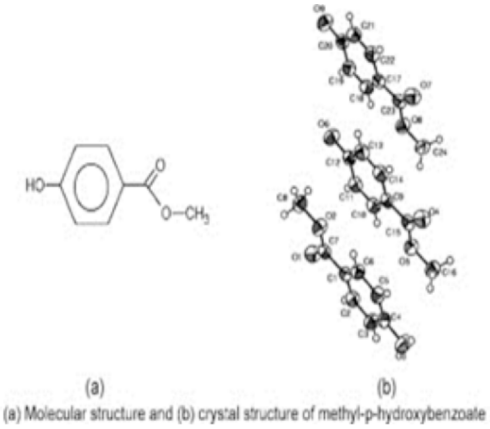

Fig.4. Molecular and crystal structure of methyl-p-hydroxy benzoate.

Review Article

New Genes and Emerging Mechanisms of Type 1 Diabetes

Aysha Karim Kiani, Asima Zia, Parveen Akhter, Sadaf Moeez, Dr Attya Bhatti, Dr Peter John
Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad Pakistan.
ayshakiani@gmail.com

Abstract

Type 1 diabetes susceptibility depends upon the complex interaction between numerous genetic as well as environmental factors. 50% of the familial clustering of T1D is explained by HLA locus alleles. Other multiple loci contribute the rest of susceptibility, in which very little were known since last few years. Four novel loci were found from the results of stage-I, genome-wide association (GWA) studies which were carried out with high-density genotyping arrays. As the stage-II of the Genome Wide Association studies completed, hopefully, most of the genetic reasons of Type 1 Diabetes will be identified.

Key words: Type 1 diabetes, Autoimmune diseases, HLA class II

Introduction

The self destruction of the pancreatic β cells by immune system leads to produce insulin at low level as a result cause Type 1 diabetes (T1D) (Devendra *et al*. 2004). Multiple genetic as well as environmental factors are one of the reasons in the onset of Type 1 Diabetes (Knip *et al*. 2005). With the entry of dendritic cells, T lymphocytes (both CD8+ and CD4+) and macrophages, destruction of insulin producing beta cells takes place, without damaging somatic cells as well as glucagon. Young individuals are mostly affected by this disease, usually most of the cases are diagnosed before age of 18 years.

Overview of T1D genetics

About half of T1D familial clusters are related to HLA region on chromosome 6p21 (Cucca *et al*. 2001; Noble *et al*. 1996; Kelly *et al*. 2003; Devendra and Eisenbarth 2003). Insulin gene (INS) located on chromosome 11p15 (Bennett *et al*. 1995; Undlien *et al*. 1995), PTPN22 located on chromosome 1p13 (Bottini *et al*. 2004; Smyth *et al*. 2004; Qu *et al*. 2005; Ladner *et al*. 2005), CTLA4 located on chromosome 2q31 (Ueda 2003), the receptor of interleukin-2 (CD25, encoded by IL2RA) that is located on chromosome 10p15 (Vella *et al*. 2005; Lowe *et al*. 2007; Qu *et al*. 2007), IFIH1 (also called MDA5) located on chromosome 2q24 (Todd *et al*. 2007; Smyth *et al*. 2006) in recent times, CLEC16A (KIAA0350) located on chromosome 16p13 (Todd *et al*. 2007; Hakonarson *et al*. 2007), PTPN2 is mapped on chromosome 18p11 and CYP27B1 located on chromosome 12q13 are crucial regions which are associated with Type 1 Diabetes (Table 1) having less effects as compared to HLA region.

The main mechanisms and the development of T1D are revealed by the study of these susceptibility genes, focusing diagnostic, therapeutic and prophetic implications. The genetic map of type 1 susceptibility genes presented by Genome Wide Association studies might enable us to distinguish between immune-dysregulation phenotypes and homogenous phenotype which react in a different way to diverse precautionary intervention. Figure 1 represents the action sites of type 1 diabetes susceptibility genes.

HLA Class II

The DQ and DR genes of the Human Leukocyte Antigen class II region contribute strongly to T1D susceptibility. DR3-DQ2 and DR4-DQ8 are most important combinations of HLA genes (haplotypes), present in 90% of Type 1 diabetic patient. However, DR15-DQ6 has found to be involved in the protection of T1D, and is found only in less then 1% patient while in general population 20% found (Devendra *et al*. 2004; Devendra and Eisenbarth 2003). Although due to linkage disequilibrium all the T1D linkage to HLA is not explained by DR-DQ, but determination of other weaker component is not easy. Minor effects from the class I HLA molecules A and B have been identified by the regression analysis recently. All nucleated cells express class I HLA genes and act as antigen presenting cells for CD8+ T cells, which are involved in autoimmune process (Nejentsev *et al*. 2007).

INS Gene:

Second most susceptible genetic locus of T1D is the VNTR mapped at 596 bp upstream of the insulin gene (INS) that is located on chromosome 11p at position 15.5. INS gene consists of 14-15 bp tandem repeat sequences (Bennett *et al.* 1995; Undlien *et al*. 1995). Short class I VNTR alleles (26 to 63 repeats) are responsible for predisposition of T1D while class III alleles (140 to 210 repeats) may exhibit protective activity. These VNTR mapped 596 bp upstream of INS regulates cis transcription of INS gene. The class III alleles are highly associated in thymus whereas show less association in INS mRNA in pancreas as compare to class I alleles (Vafiadis *et al*. 1997; Pugliese *et al*. 1997).

PTPN22:

Recently a third T1D susceptibility gene PTPN22 mapped on chromosome 1p13 has been identified (Bottini *et al*. 2004; Smyth *et al*. 2004; Qu *et al*. 2005; Ladner *et al*. 2005) which is directly associated with T cell activation. PTPN22 encodes protein lymphoid tyrosine phosphatase which is also called as 'Lyp'. This protein dephosphorylate the three kinases which are important for TCR signaling

Corresponding Author: Aysha Karim Kiani

Table 1. T1D-susceptibility loci

Locus	Gene	Odds ratio		Predisposing allele frequency	Postulated mechanism	Refs
		Het	Hom			
6p21	*HLA DR-DQ*	0.02–11.4	0.02–49.2	~20% of Europeans carry at least one predisposing allele and ~15% a strongly protective one	Present exogenous antigen processed by APCs, with some antigen specificity. Predisposing alleles might bind autoantigens poorly, compromising adaptive self-tolerance.	[9–13,31–34,70]
6P21	*HLA-A*	0.29–1.23		Multiple alleles	Present endogenously synthesize antigen (e.g. viral) by all cells. Mechanism might be similar to DQ-DR.	[30]
6p21	*HLA-B*	0.73–3.6		Multiple alleles		[30]
11p15	*INS*	2.68	3.27	0.71	Modulation of thymic expression and central tolerance to insulin.	[14–16,35–38,41,42]
1p13	*PTPN22*	1.95	4.16	0.94	Moderates TCR signaling by dephosphorylation. Gain of function might inhibit proper development of tolerance.	[17–21,43–50,71]
2q31	*CTLA4*	1.14	1.5	0.55	Moderates T-cell activation. Functional effect of locus to be determined.	[22,23, 51–55]
10P15	*IL2RA*	1.87	3.89	0.90	Modulation of the effect of IL2 on regulatory and/or effector T lymphocytes.	[24–26]
2q24	*IFIH1*	1.18	1.37	0.61	Triggers interferon response upon recognition of viral RNA. Might be involved in infectious etiology of T1D.	[50–53]
16p13	*CLEC16A*	1.29	1.42	0.68	Function unknown. Contains C-lectin and ITAM domains.	[12–14]
18q11	*PTPN2*	1.33	1.61	1.7	Phosphotyrosine phosphatase. Role likely similar to *PTPN22*.	[13,14]
12q24		1.24	1.74	0.48	Not mapped to specific gene.	[13,14]
12q13		1.31	1.58	0.35		[13,14]

hence inhibiting its signal transduction activity (Hill *et al*. 2002; Gregersen and Behrens 2006). Lyp also interact with kinases suppressor called Csk: C-terminal Src tyrosine kinase which results in the downregulaton of T cell activation (Gregersen and Behrens 2006; Cohen *et al*. 1999). T1D associated SNP at position 1858 from Cytosine to Thymine results in the substitution of arginine to tryptophan at location 620 of lymphoid tyrosine phosphatase protein. As tyrosine phosphatases protein play a significant role in T cell receptor signaling, hence PTPN22 is considered a good candidate gene for T1D susceptibility. PEST domain-enriched tyrosine phosphatase commonly called Pep, which is the murine homolog of Lyp, when specifically disrupted cause increased number of memory T cells that emphasize autoimmunity (Hasegawa 2004). When Lyp interacts with tyrosine kinase Csk its function of inhibiting T cell receptors transduction is greatly improved (Cloutier and Veillette 1999).

CTLA4

CTLA4 (CYTOTOXIC T-LYMPHOCYTE-ASSOCIATED ANTIGEN 4) mapped on chromosome 2q33 is also know to be a good candidate gene for type 1 diabetes as it negatively regulate T cell activation. In one of the largest genomic studies up till now (Ueda 2003), effect of flanking region of 3' end of the SNP was mapped, however, 5' effect of gene cannot be excluded. In one of the 5' effect substitution of A to G at residue 49 occur in first exon, resulting in the replacement of Ala for Thr, while C318T substitution occure in promoter region. (Anjos *et al*. 2004; Anjos and Polychronakos 2004; Teft *et al*. 2006). Primary amino acid sequence of CTLA4 is altered by A49G substitution only. Unexpected glycosylation of CTLA4 mutant in endoplasmic reticulum, as observed by A49G CTLA4 in vitro studies, cause reduction in its cell-surface expression (Anjos *et al*. 2002). On the other hand increased level of CTLA4 expression (Wang *et al*. 2002; Anjos and Polychronakos 2004) is observed in C318T polymorphism due to higher promoter activity, leading to decrease activation level of T cell. Therefore C318T polymorphism play a defensive role for autoimmune disease.

IL2RA

From recently conducted genome wide association studies a novel type 1 diabetes locus IL2RA has been identified, mapped on chromosome 10p15. 1 (Vella *et al*. 2005; Lowe *et al*. 2007; Qu *et al*. 2007). Three α chains of IL-2 receptor complex, which is also called CD25, are encoded by the eight exons of IL2RA gene. IL2RA is an essential component of immune regulation as it modulate immunity. For the suppression of autoimmune disease and T cell mediated immune response, expression of IL2RA on regulatory T cell is very essential. (Salomon *et al*. 2000; Malek and Bayer 2004; Viglietta *et al*. 2004). Due to these functions IL2RA gene is considered as an appealing candidate for Type 1 diabetes, which plays an important role in its pathogenesis, involving regulatory T cells.

IFIH1

Innate immunity regulating RNA helicase, against viral infection as well as various autoimmune conditions, is encoded by interferon-induced helicase (IFIH1) mapped on chromosome 2q24 (Kato *et al*. 2006). As a result of major association study of candidate SNPs, IFIH1 which is also called Helicard, gene and MDA-5 (melanoma differentiation-associated 5) (Smyth *et al*. 2006) is identified as a new locus for Type 1 Diabetes. Even with the presence of other genes in LD block, interferon-induced helicase (IFIH1) is consider as the best candidate for type 1 Diabetes as it is only gene with some common known nonsynonymous polymorphism. Role of interferon-induced helicase (IFIH1) in the protection of host from infection that is caused by virus by recognizing nucleic acid of virus and activating apoptotic and cellular antiviral response is thought to be one of its major aspect. (Yoneyama *et al*. 2005; Meylan *et al*. 2006). As it is suggested by many studied that there is a correlation between T1D and infection caused by virus (Knip *et al*.

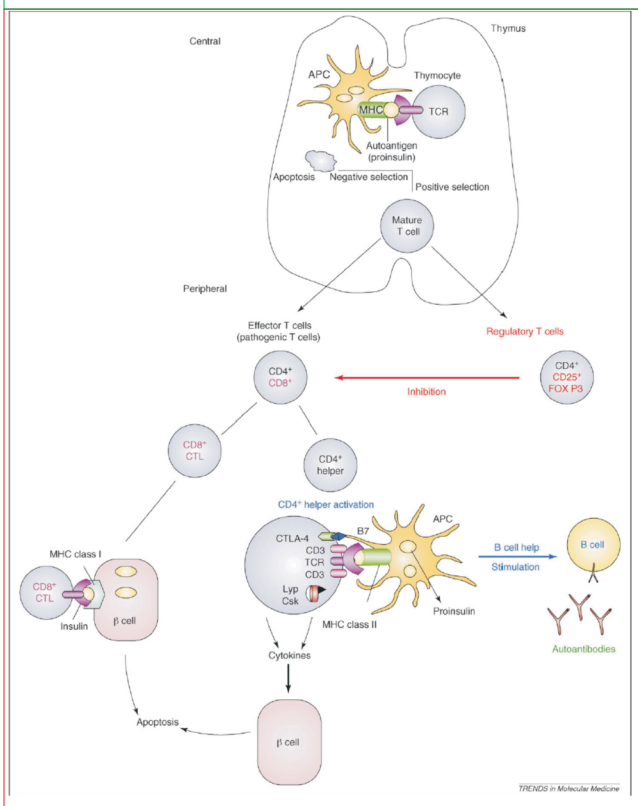

Fig. 1. Peripheral and Central tolerance to self peptides. The thymocyte maturation and its selection taking place in thymus results in central tolerance. The process engages MHC proteins and APC's interaction, the self peptide (proinsulin) and the TCR on thymocyte. Negative selection of strongly self reactive thymocytes take place (98%) and thymocytes with low affinity are positively selected (2%).

2005), hence the importance of interferon-induced helicase (IFIH1) gene as a good functional candidate of T1D is well considered. The SNP rs1990760 is supposed to be the most associated marker of IFIH1, and encode Alanine to threonine substitution at codon 946 (Smyth *et al*. 2006). As a result of these findings related to IFIH1 gene, type of pathogens which are potentially involved in triggering type 1 diabetes are somewhat narrowed down.

CYP27B1

Vitamin D 1-α-hydroxylase, which plays an important role in the synthesis of active vitamin D is encoded by CYP27B1: subfamily 27, cytochrome p450 and polypeptide 1. There are two single nucleotide polymorphisms in perfect LD (–1260C > A and +2838T > C) were establish to be related with Type 1 Diabetes (Bailey *et al*. 2007). As there is no splicing variant or common amino acid polymorphism, hence it is suggested that it probably affects the transcription mechanism. Various epidemiological evidences supporting the idea that vitamin D supplementation might be used to prevent Type 1 Diabetes has proved its significant importantance (EURODIAB 1999; Hyppo¨nen *et al*. 2001).

CLEC16A

One locus was identified in several populations by using different techniques (Todd *et al*. 2007; Hakonarson *et al*. 2007). It is located to a 300 kb LD block on chromosome 16p at position 13.2 and it contains a single gene. This gene is known as C-type lectin domain family 16 gene A (CLEC16A), formerly called as KIAA0350). It is expressed in immune cells as well as encodes a sequence of protein that is predicted having a C-type lectin domain (Finn *et al*. 2006). It also expresses in specialized APC like Dendritic Cells and B lymphocytes as well as in Natural Killer cells. This is very fascinating as C-type lectins participate in up taking of antigen and its presentation by DCs and b cells. There is a possibility that there is no association between T1D and CLEC16A but variants are affecting other two genes that slightly overlap the related LD block. Genes that were predicted theoretically and have't been studied so far are LOC729954 and dexmethasone induced (DEXI) that is upregulated in emphysema (Tafuri *et al*. 2001). Effects on the immune system that is caused by glucocorticoids usually make DEXI as a interesting candidate, but the problem associated with it is that it is usually expressed in low levels in heart, lung, liver and brain [GNF SymAtlas (http://symatlas.gnf.org/ SymAtlas)].

PTPN2

GWA studies present a novel locus which is located on chromosome 18q11 and is known as Phosphotyrosine-protein phosphatase non-receptor (PTPN2) (Todd *et al*. 2007) PTPN2 is stands for phosphotyrosine protein phosphatase, non-receptor 2. Tyrosine phosphorylation that occur in activation of lymphocytes elucidate the importance of this gene in pathogenesis of T1D which may results in designing novel therapies and patho-physiological insights by some special types of inhibitors.

Genome Wide Association studies have revealed high level of statistical significance in only a little percentage of loci, leading to replication. It is hoped that as the replication stages completed it will augment additional groups that will improve statistical power. To reveal actual genetic basis of T1D will take some time. Molecular basis of T1D will be revealed with the help of fine mapping and functional studies that will also help in designing novel therapies.

Conclusions and Future Perspectives

T1D is a multifaceted and polygenic disease. Identification of T1D susceptibility genes is hindered by the multi-factorial character of the disease and genetic interactions that occur between loci. Up till now, HLA-DQ genes, located in HLA region are the most susceptible genes in T1D. MHC genes may modify their influence (i.e. HLA-B, -DRB1 and -DPB1). The VNTR of insulin gene also play crucial role in this disease. Genome wide screening has identified many regions of chromosomes which may contain genes that are susceptible to T1D, though its location and aetiological mutations or polymorphisms have to be identified.

More genetic study is needed in order to recognize all the T1D susceptible genes as well as to identify their role in pathogenesis of disease. This type of information will help to develop the screening approaches to identify those individuals that are higher at risk to develop T1D. This information will facilitate us to develop strategies for treatment. Thus, genetics understanding of T1D will make a significant contribution for the prevention of the disease.

REFERENCES

1. Anjos S and Polychronakos C. 2004. Mechanisms of genetic susceptibility to type I diabetes: beyond HLA. Mol. Genet. Metab, 81: 187–195.
2. Anjos SM, Nguyen A, Ounissi-Benkalha H, Tessier MC, Polychronakos C. 2002. A common autoimmunity predisposing signal peptide variant of the cytotoxic T-lymphocyte antigen 4 results in inefficient glycosylation of the susceptibility allele. J. Biol. Chem, 277: 46478–46486.
3. Anjos SM, Tessier Mc, Polychronakos C. 2004. Association of the cytotoxic T lymphocyte associated antigen 4 gene with type 1 diabetes: evidence for independent effects of two polymorphisms on the same haplotype block. J. Clin. Endocrinol. Metab, 89: 6257–6265.
4. Bailey R, Cooper JD, Zeitels L, Smyth DJ, Yang JH, Walker NM, Hyppönen E, Dunger DB, Ramos-Lopez E, Badenhoop K, Nejentsev S, Todd JA. 2007. Association of the vitamin D metabolism gene CYP27B1 with type 1 diabetes. Diabetes, 56: 2616–2621.
5. Bennett ST, Lucassen AM, Gough SC, Powell EE, Undlien DE, Pritchard LE, Merriman ME, Kawaguchi Y, Dronsfield MJ. 1995. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. Nat. Genet, 9: 284–292.
6. Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K, Rostamkhani M, MacMurray J, Meloni GF, Lucarelli P, Pellecchia M, Eisenbarth GS, Comings D, Mustelin T. 2004. A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nat. Genet, 36: 337–338.
7. Cloutier JF and Veillette A. 1999. Cooperative inhibition of T-cell antigen receptor signaling by a complex between a kinase and a phosphatase. J. Exp. Med. 189: 111–121.
8. Cohen S, Dadi H, Shaoul E, Sharfe N, Roifman CM. 1999. Cloning and characterization of a lymphoidspecific, inducible human protein tyrosine phosphatase. Lyp. Blood, 93: 2013–2024.
9. Cucca F, Lampis R, Congia M, Angius E, Nutland S, Bain SC, Barnett AH, Todd JA. 2001. A correlation between the relative predisposition of MHC class II alleles to type 1 diabetes and the structure of their proteins. Hum. Mol. Genet, 10: 2025–2037.
10. Devendra D, and Eisenbarth GS. 2003. Immunologic endocrine disorders. J. Allergy Clin. Immunol, 111: 624-636.
11. Devendra D, Liu E, Eisenbarth GS. 2004. Type 1 diabetes: recent developments. BMJ, 328: 750–754.
12. EURODIAB. 1999. Vitamin D supplement in early childhood and risk for type I (insulin-dependent) diabetes mellitus: the EURODIAB substudy 2 study group. Diabetologia, 42: 51–54.
13. Finn RD, Mistry J, Schuster-Böckler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer EL, Bateman A. 2006. Pfam: clans, web tools and services. Nucleic Acids Res, 34: D247–D251.
14. Gregersen PK and Behrens TW. 2006. Genetics of autoimmune diseases disorders of immune homeostasis. Nat. Rev. Genet, 7: 917–928.
15. Hakonarson H, Grant SFA, Bradfield JP, Marchand L, Kim CE, Glessner JT, Grabs R, Casalunovo T, Taback SP, Frackelton EC, Lawson ML, Robinson LJ, Skraban R, Lu Y, Chiavacci RM, Stanley CA, Kirsch SE, Rappaport EF, Orange JS, Monos DS, Devoto M, Qu HQ, Polychronakos C. 2007. A genome-wide association study identifies KIAA0350 as a type 1 diabetes gene. Nature, 448: 591–594.
16. Hasegawa K. 2004. PEST domain-enriched tyrosine phosphatase (PEP) regulation of effector/memory T cells. Science, 303: 685–689.
17. Hill RJ, Zozulya S, Lu YL, Ward K, Gishizky M, Jallal B. 2002. The lymphoid protein tyrosine phosphatase Lyp interacts with the adaptor molecule Grb2 and functions as a negative regulator of T-cell activation. Exp. Hematol, 30: 237–244.
18. Hyppo¨nen E, Läärä E, Reunanen A, Järvelin MR, Virtanen SM. 2001. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. Lancet, 358: 1500–1503.
19. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature, 441: 101–105.
20. Kelly MA, Rayner ML, Mijovic CH, Barnett AH. 2003. Molecular aspects of type 1 diabetes. Mol. Pathol, 56: 1–10.
21. Knip M, Veijola R, Virtanen SM, Hyoty H, Vaarala O, Akerblom HK. 2005. Environmental triggers and determinants of type 1 diabetes. Diabetes, 54 (Suppl. 2): S125–S136.
22. Ladner MB, Bottini N, Valdes AM, Noble JA. 2005. Association of the single nucleotide polymorphism C1858T of the PTPN22 gene with type 1 diabetes. Hum. Immunol, 66: 60–64.
23. Lowe CE, Cooper JD, Brusko T, Walker NM, Smyth DJ, Bailey R, Bourget K, Plagnol V, Field S, Atkinson M, Clayton DG, Wicker LS, Todd JA. 2007. Large-scale genetic fine mapping and genotype phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. Nat. Genet, 39: 1074–1082.
24. Malek TR and Bayer AL. 2004. Tolerance, not immunity, crucially depends on IL-2. Nat. Rev. Immunol, 4: 665–674.

25. Meylan E, Tschopp J, Karin M. 2006. Intracellular pattern recognition receptors in the host response. Nature, 442: 39–44.
26. Nejentsev S, Howson JMM, Walker NM, Szeszko J, Field SF, Stevens HE, Reynolds P, Hardy M, King E, Masters J, Hulme J, Maier LM, Smyth D, Bailey R, Cooper JD, Ribas G, Campbell RD, Clayton DG, Todd JA. 2007. Wellcome Trust Case Control Consortium. Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A. Nature, 450: 887–892.
27. Noble JA, Valdes AM, Cook M, Klitz W, Thomson G, Erlich HA. 1996. The role of HLA class II genes in insulin dependent diabetes mellitus: molecular analysis of 180 Caucasian, multiplex families. Am. J. Hum. Genet, 59:1134–1148.
28. Pugliese A, Zeller M, Fernandez A Jr, Zalcberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST, Patel DD. 1997. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat. Genet, 15: 293–297.
29. Qu H, Tessier MC, Hudson TJ, Polychronakos C. 2005. Confirmation of the association of the R620W polymorphism in the protein tyrosine phosphatase PTPN22 with type 1 diabetes in a family based study. J. Med. Genet, 42: 266–270.
30. Qu HQ, Montpetit A, Ge B, Hudson TJ, Polychronakos C. 2007. Toward further mapping of the association between the IL2RA locus and type 1 diabetes. Diabetes, 56: 1174–1176.
31. Salomon B. Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity, 12: 431–440.
32. Smyth D, Cooper JD, Collins JE, Heward JM, Franklyn JA, Howson JMM, Vella A, Nutland S, Rance HE, Maier L, Barratt BJ, Guja C, Ionescu-T□rgovişte C, Savage DA, Dunger DB, Widmer B, Strachan DP, Ring SM, Walker N, Clayton DG, Twells RCJ, Gough STL, Todd JA. 2004. Replication of an association between the lymphoid tyrosine phosphatase locus (LYP/PTPN22) with type 1 diabetes, and evidence for its role as a general autoimmunity locus. Diabetes, 53: 3020–3023.
33. Smyth DJ, Cooper JD, Bailey R, Field S, Burren O, Smink LJ, Guja C, Tirgoviste CI, Widmer B, Dunger DB, Savage DA, Walker NM, Clayton DG, Todd JA. 2006. A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. Nat. Genet, 38: 617–619.
34. Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, Boucher LM, Bouchard D, Chan VS, Duncan G, Odermatt B, Ho A, Itie A, Horan T, Whoriskey JS, Pawson T, Penninger JM, Ohashi PS, Mak TW. 2001. ICOS is essential for effective T-helper-cell responses. Nature, 409: 105–109.
35. Teft WA, Kirchhof MG, Madrenas J. 2006. A molecular perspective of CTLA-4 function. Annu Rev Immunol, 24: 65–97.
36. Todd, J.A. , Walker NM, Cooper JD, Smyth JD, Downes K, Plagnol V, Bailey R, Nejentsev S, Field SF, Payne F, Lowe CE, Szeszko JS, Hafler JP, Zeitels L, Yang JHM, Vella A, Nutland S, Stevens HE, Schuilenburg H, Coleman G, Maisuria M, Meadows W, Smink LJ, Healy B, Burren OS, Lam AAC, Ovington NR, Allen J, Adlem E, Leung HT, Wallace C, Howson JMM, Guja C, Ionescu-Tîrgovişte C, Simmonds MJ, Heward JM, Gough SCL, Dunger DB, Wicker LS, Clayton DG. 2007. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. Nat. Genet, 2007: 857–864.
37. Ueda H. 2003. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature, 423: 506–511.
38. Undlien DE, Bennett ST, Todd JA, Akselsen HE, Ikäheimo I, Reijonen H, Knip M, Thorsby E, Rønningen KS. 1995. Insulin gene region-encoded susceptibility to IDDM maps upstream of the insulin gene. Diabetes, 44: 620–625.
39. Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E, Polychronakos C.. 1997. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. Nat. Genet, 15: 289–292.
40. Vella A, Cooper JD, Lowe CE, Walker N, Nutland S, Widmer B, Jones R, Ring SM, McArdle W, Pembrey ME, Strachan DP, Dunger DB, Twells RCJ, Clayton DG, Todd JA. 2005. Localization of a type 1 diabetes locus in the IL2RA/CD25 region by use of tag single-nucleotide polymorphisms. Am. J. Hum. Genet, 76: 773–779.
41. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. 2004. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J. Exp. Med, 199: 971–979.
42. Wang XB, et al. 2002. A CTLA4 gene polymorphism at position -318 in the promoter region affects the expression of protein. Genes Immun, 3: 233–234.
43. Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M Jr, Akira S, Yonehara S, Kato A, Fujita T. 2005. Shared and unique functions of the DExD/ H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol, 175: 2851–2858.

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6P21	*HLA-A*	0.29–1.23		Multiple alleles	Present endogenously synthesize antigen (e.g. viral) by all cells. Mechanism might be similar to DQ-DR.	[30]
6p21	*HLA-B*	0.73–3.6		Multiple alleles		[30]
11p15	*INS*	2.68	3.27	0.71	Modulation of thymic expression and central tolerance to insulin.	[14–16,35–38,41,42]
1p13	*PTPN22*	1.95	4.16	0.94	Moderates TCR signaling by dephosphorylation. Gain of function might inhibit proper development of tolerance.	[17–21,43–50,71]
2q31	*CTLA4*	1.14	1.5	0.55	Moderates T-cell activation. Functional effect of locus to be determined.	[22,23, 51–55]
10P15	*IL2RA*	1.87	3.89	0.90	Modulation of the effect of IL2 on regulatory and/or effector T lymphocytes.	[24–26]
2q24	*IFIH1*	1.18	1.37	0.61	Triggers interferon response upon recognition of viral RNA. Might be involved in infectious etiology of T1D.	[50–53]
16p13	*CLEC16A*	1.29	1.42	0.68	Function unknown. Contains C-lectin and ITAM domains.	[12–14]
18q11	*PTPN2*	1.33	1.61	1.7	Phosphotyrosine phosphatase. Role likely similar to *PTPN22*.	[13,14]
12q24		1.24	1.74	0.48	Not mapped to specific gene.	[13,14]
12q13		1.31	1.58	0.35		[13,14]

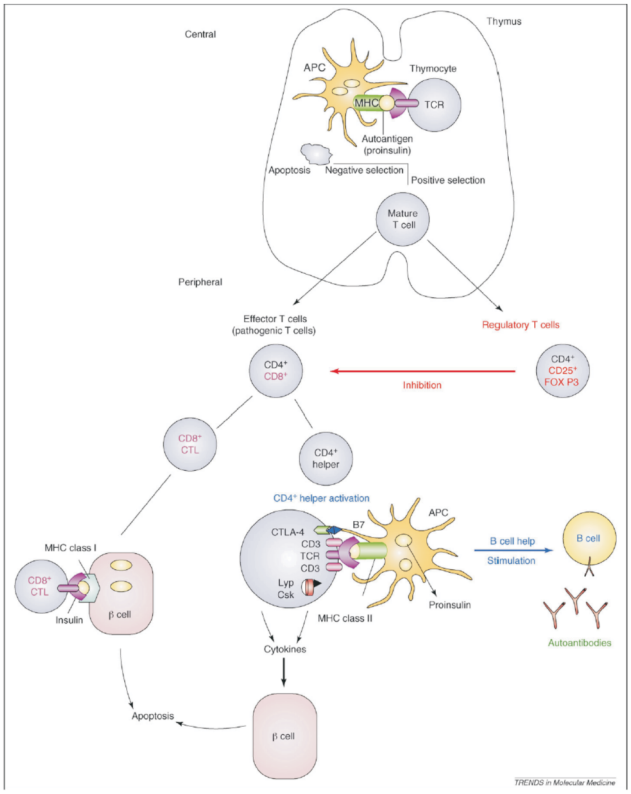

Fig. 1. Peripheral and Central tolerance to self peptides. The thymocyte maturation and its selection taking place in thymus results in central tolerance. The process engages MHC proteins and APC's interaction, the self peptide (proinsulin) and the TCR on thymocyte. Negative selection of strongly elf reactive thymocytes take place (98%) and thymocytes with low affinity are positively selected (2%).